

UNIVERSITY OF ALBERTA

**The Isolation of a *Pto* Homolog in *Brassica napus* L. Using a
PCR-Based Approach and the Characterization of *B. napus* L.
as a New Host for *Pseudomonas syringae* pv. *tomato***

BY



BRET WAYNE KENNEDY

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fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

IN

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DEPARTMENT OF BIOLOGICAL SCIENCES

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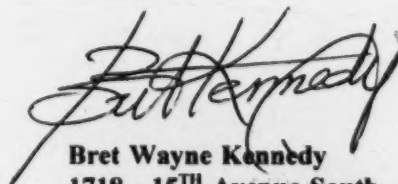
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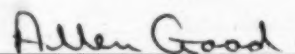
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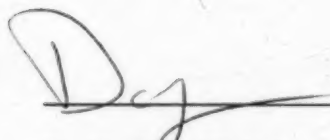
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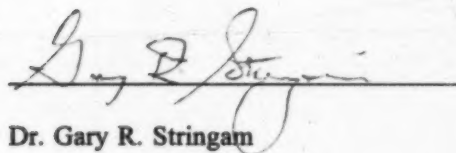
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ABSTRACT

Degenerate oligonucleotide primers and PCR were used to amplify a *Pto* homolog from *Brassica napus* L. This positive amplification of a candidate resistance gene illustrates the potential of using a heterologous PCR approach to identify and clone resistance genes from diverse plant taxa. RFLP analysis was also employed to identify an *RPS2* homolog in the crop brassicas. However, the required genetic criteria could not be met in order to prove that these resistance gene homologs were functional genes rather than pseudogenes. Sequence analysis of the *Pto* homolog indicated that it and six other plant proteins may constitute a new family of serine/threonine kinases.

I was also able to definitively show that the tomato pathogen, *Pseudomonas syringae* pathovar *tomato*, is capable of causing an infectious disease in the crucifer, *B. napus* L. This finding lends support to the concept that *P. syringae* pathovars *maculicola* and *tomato* may constitute a single genetic group.

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LIST OF ABBREVIATIONS

1C	the unreplicated haploid genome of a species
<i>avr</i> gene(s)	avirulence gene(s)
Avr protein(s)	<i>avr</i> gene product(s)
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
CTAB	cetyltrimethylammonium bromide
cv(s).	cultivar(s)
dCTP	deoxycytidine-5'-triphosphate
ddH ₂ O	double distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EREBPs	ethylene-responsive element-binding proteins
F ₁	first filial generation
F ₂	second filial generation
f. sp.	forma specialis
GI	Genbank Identifier
HCTR	HC-toxin reductase
H ₂ O ₂	hydrogen peroxide
HR	hypersensitive response
<i>hrp</i> gene(s)	hypersensitivity reaction and pathogenicity gene(s)
Hrp type III protein secretion system	a protein secretion system composed of <i>hrp</i> gene products
IL-1R	interleukin-1 receptor
kbp	kilobase pair
KOH	potassium hydroxide
L.	Linnaeus

LB-Miller	Luria-Bertani-Miller
LRR(s)	leucine-rich repeat(s)
LZ(s)	leucine zipper(s)
Mbp	megabase pair
NADPH	the reduced form of the coenzyme, nicotinamide adenine dinucleotide phosphate
NBS(s)	nucleotide binding site(s)
NBY	nutrient broth-yeast extract
O ₂	molecular oxygen
O ₂ ⁻	superoxide anion
·OH	hydroxyl radical
ORF(s)	open reading frame(s)
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogenesis-related
<i>Pst</i> DC3000	<i>Pseudomonas syringae</i> pv. <i>tomato</i> strain DC3000
pv.	pathovar
<i>R</i> gene(s)	plant resistance gene(s)
<i>R</i> protein(s)	<i>R</i> gene product(s)
RADP	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TIR domain	Toll-IL-1R homology region
T _m	melting temperature
TMV	Tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)

v/v	percent "volume in volume"
V	volt
var.	variety
VB-tar	Vogel-Bonner-tartrate
w/v	percent "weight in volume"

1. INTRODUCTION

In the last five years a number of disease resistance genes have been cloned and characterized in numerous plant species. These plant disease resistance genes have been shown to confer resistance to a wide range of pathogens including bacteria, fungi, nematodes, and viruses. In addition, the protein products of these plant disease resistance genes can be classified into five distinct categories based on striking structural similarities. This homology between these gene products indicates that signalling events within the plant defense response pathway may be evolutionarily conserved among the species in the plant kingdom (Baker et al., 1997).

Incompatible interactions between plants and pathogens depend on the interaction of resistance genes in plants and avirulence genes in phytopathogens. The avirulence genes of a pathogen mediate the elicitation of the hypersensitive response and resistance only in plants that possess corresponding resistance genes. Therefore, the possession of avirulence genes results in specific recognition of the pathogen by certain plant species or cultivars (Staskawicz et al., 1984; Keen, 1990; Goodman and Novacky, 1994; He, 1996). This interaction is known as the "gene-for-gene" model and it predicts that plant resistance is governed by the interaction of single dominant host genes for resistance and the corresponding, dominant pathogen genes for avirulence (Leach and White, 1996; Hammond-Kosack and Jones, 1997).

At the time this project was initiated, the only plant disease resistance gene conforming to the gene-for-gene model to be cloned and characterized was the tomato *Pto* gene (Martin et al., 1993a). *Pto* confers resistance to the bacterial pathogen *Pseudomonas syringae* pathovar (pv.) *tomato* and it encodes a functional serine/threonine protein kinase (Martin et al., 1993a; Loh and Martin, 1995a; Loh and Martin, 1995b; Rommens et al., 1995a). Since *Pto*-mediated resistance conforms to the gene-for-gene model, it only confers resistance to *P. syringae* pv. *tomato* races that express the avirulence gene, *avrPto* (Ronald et al., 1992).

I attempted to identify and characterize a *Pto* homolog in the crop brassicas because Martin et al. (1993a) were able to identify *Pto* homologs in *Arabidopsis*

thaliana. The presence of *Pto* homologs in *A. thaliana* suggested that *Pto* homologs are likely to exist in the crop brassicas since *A. thaliana* belongs to the same family (family *Cruciferae*) as these *Brassica* species. Additional factors that prompted the search for a *Pto* homolog in the crop brassicas were the fact that there are numerous genes encoding serine/threonine protein kinases present in eukaryotic genomes (Hanks et al., 1988; Taylor et al., 1993; Hanks and Hunter, 1995) and an uncharacterized strain of *P. syringae* is known to be an infectious agent of canola (Henry, 1974; Henry and Letal, 1977).

The term "canola" is used to describe cultivated varieties of *Brassica napus* and *Brassica rapa* (family *Cruciferae*) that have low erucic acid content in the extracted seed oil and low glucosinolate content in the residual meal (Downey and Rakow, 1991; Downey and Rimmer, 1993). Canola is grown extensively in Canada as a source of edible vegetable oil and, due to its multi-million dollar profits, canola has become a very agronomically important crop. As a result, I wanted to use the information obtained from the cloning of *Pto* to identify resistance gene homologs in canola and the related crop brassicas. A polymerase chain reaction (PCR)-based approach was utilized to determine if the well-established strategy of PCR with degenerate oligonucleotides could be employed to identify uncharacterized disease resistance genes in distantly related crops.

For this attempt at cloning a *Pto* homolog in canola to work, I also needed to demonstrate that *P. syringae* pv. *tomato* was a causal agent of disease in *B. napus* or *B. rapa*. Moreover, if a *Pto* homolog existed in canola it would be necessary to establish that *P. syringae* pv. *tomato* strains expressing the *avrPto* gene were incapable of causing disease in canola. These pathogenicity experiments had to be performed since these plant species had never before been characterized as hosts for *P. syringae* pv. *tomato*. In addition, after cloning a *Pto* homolog I would need to demonstrate the cosegregation of the homolog with a phenotypically defined *P. syringae* pv. *tomato* resistance gene in canola. This segregation analysis would provide genetic evidence that verified the presence of a functional *Pto* homolog in canola.

2. LITERATURE REVIEW

2.1 SIGNIFICANCE OF PLANT DISEASES

Plant-pathogen interactions have had a profound impact on the development of civilization since the moment humans began the cultivation of wild plant species for food. Ancient records of famines, plagues, and epidemics describe serious plant diseases such as smuts, rusts, and mildews (Jackson and Taylor, 1996). The death of a quarter million people during the Irish potato famine of the 1840s, and the suffering of a countless number of underfed people living in underdeveloped countries are more recent examples of plant diseases. Although there have been several recent advances in plant pathology, destruction of food resources by diseases still frequently occurs resulting in malnutrition, starvation, migration, and death of people and animals (Agrios, 1988).

Currently, agriculture is a multi-billion dollar industry in Canada. Specifically, it has been estimated that over \$9 billion annually is derived from field crops (Martens et al., 1988) and approximately \$1 billion from vegetable crops (Howard et al., 1994), making the agricultural industry a vital part of the Canadian economy.

When plant diseases cause even minimal crop losses, the producers and the economy are negatively affected. If a severe disease epidemic strikes a major crop, the losses can be catastrophic to those who are directly involved with the growth, manufacture, and distribution of plants and plant products. Since we are all consumers of plants and of the infinite number of products derived from plants, it can be said that every citizen of Canada is affected by plant diseases and their associated crop losses (Agrios, 1988; Martens et al., 1988).

2.2 INCOMPATIBLE PLANT-PATHOGEN INTERACTIONS

2.2.1 *General aspects of plant HR*

Stakman (1915) was the first researcher to use the term hypersensitive reaction

to describe a resistant plant response in various grasses upon inoculation with an incompatible fungus. This response involved a remarkably rapid cell death of host tissues infected with the fungal pathogen *Puccinia graminis* (Goodman and Novacky, 1994). The term hypersensitive response (HR) is now the customary expression used to describe this host cell response (He, 1996).

Half of a century after Stakman (1915) discovered the fungus-induced HR, Klement et al. (1964) established that phytopathogenic bacteria were also capable of inducing a plant HR. Specifically, these researchers were the first to demonstrate the effect of different bacteria in defining the nature of the plant response to incompatible (avirulent), compatible (virulent), and saprophytic bacteria. They artificially inoculated tobacco leaves with various bacterial species and recorded three primary plant responses (Klement et al., 1964).

First, Klement et al. (1964) observed an HR in which a rapid death of plant cells occurred 12 to 24 hours after bacterial inoculation. This cell death was followed by tissue desiccation and bacteria did not spread to surrounding tissues. Multiple avirulent pathovars of *P. syringae* induced this reaction. The second observable response was a disease reaction involving a delayed host cell response, with bacteria spreading to other, uninoculated tissues of the plant. The HR symptoms did not develop but, in contrast, necrotic lesions characteristic of bacterial disease gradually developed and enlarged. This reaction was induced by the virulent bacterium *P. syringae* pv. *tabaci* and resulted in the wildfire disease of tobacco. The final response these researchers observed was no noticeable reaction after they inoculated the saprophytic bacterium, *Pseudomonas fluorescens*, onto tobacco leaves (Klement et al., 1964). These tobacco plant responses to compatible, incompatible, and saprophytic pseudomonads have been corroborated by subsequent studies on numerous other plants inoculated with their own specific bacterial pathogens.

The HR can occur when plants are challenged by bacteria, fungi, viruses, or nematodes and it is one of the most important defense mechanisms of plants. When the HR occurs, it is consistently associated with plant resistance and only occurs in interactions between incompatible (avirulent) pathogens and host plants. In such an

interaction there is no observable difference between the way the bacterial pathogen penetrates a resistant or susceptible plant. However, there is a marked difference between the tissue responses of resistant and susceptible varieties after infection and invasion by a phytopathogenic bacteria. The infected cells of resistant varieties rapidly lose turgor, turn brown, and die. In contrast, the infected cells of susceptible varieties survive significantly longer prior to becoming necrotic (Agrios, 1988).

Therefore, there are two types of plant cell death associated with bacterial infection of plants. One category of cell death comprises rapid, hypersensitive cell death localized at the site of infection and occurs during an incompatible interaction involving a resistant plant and an avirulent bacterial pathogen. The other type of cell death is a slow, delayed response that spreads beyond the initial site of infection. This cell death response develops during some compatible interactions between a susceptible plant and a virulent, necrogenic bacterial pathogen (He, 1996).

Within hours of inoculating a plant with an HR-inducing bacterial pathogen, the tissue becomes flaccid as turgor pressure is lost and the infected tissue continues to wilt until it eventually collapses. This complete desiccation of infected tissue occurs within 24 hours (Goodman and Novacky, 1994). In addition to tissue desiccation, hypersensitive cell death is associated with the induction of multifaceted defense responses. For example, numerous physiological changes occur in the infected cells and the cells surrounding them.

These physiological changes include loss of permeability of cell membranes; ion fluxes; cellular decompartmentalization; cross-linking and strengthening of plant cell wall; increased respiration; accumulation and oxidation of phenolic compounds; production of reactive oxygen species and phytoalexins (antimicrobial compounds); and induction of numerous defense-related genes encoding 'pathogenesis-related' (PR) proteins. Within the tissue expressing the HR and within the surrounding cells, bacterial pathogens are isolated by necrotic tissue, the bacterial population declines, and any surviving bacteria are confined to the site of inoculation. The final result of the HR is the death and collapse of the infected and surrounding cells resulting in resistance to the invading pathogen (Agrios, 1988; Lamb et al., 1989; Dixon et al.,

1994; Goodman and Novacky, 1994; He, 1996).

The plant HR towards an incompatible pathogen can be considered a sacrifice of locally infected tissue to prevent the spread of the pathogen into healthy plant tissues. Unlike the HR, the slow, delayed cell death that spreads beyond the initial site of infection during a compatible interaction between a susceptible plant and a virulent, necrogenic bacterial pathogen does not effectively prevent pathogen multiplication or spread. In a compatible interaction with a virulent pathogen the HR does not occur. Additionally, the associated defense responses may either not occur or, if they do occur, their induction is slower and/or weaker. Therefore, this type of cell death is not associated with local plant resistance and disease develops (Agrios, 1988; Lamb et al., 1989; Staskawicz et al., 1995; He, 1996).

2.2.2 The oxidative burst in plant defense

It was initially reported in 1983 that the generation of superoxide anions and hydrogen peroxide represented a novel plant defense response distinct from transcription based defense mechanisms (Doke, 1983a; Doke, 1983b). The rapid generation of reactive oxygen species (ROS) has now been reported in numerous plant-pathogen interactions and is a characteristic feature of the HR. In fact, the production of ROS is, typically, the first response activated in many incompatible interactions and it may be the trigger that initiates the HR (Hammond-Kosack and Jones, 1996). ROS are toxic intermediates that are produced from the progressive reduction of molecular oxygen (O_2) and the principal species involved in plant-pathogen interactions are the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide molecule (H_2O_2), and hydroxyl radical ($\cdot OH$) (Mehdy, 1994).

Researchers have proposed several roles for the function of the oxidative burst and ROS production. One possibility is that $O_2^{\cdot-}$, H_2O_2 , and other ROS could be directly toxic to invading pathogens (Keppler and Baker, 1989; Peng and Kuc, 1992). The strengthening of the plant cell wall to pathogen attack by oxidative cross-linking of the cell wall is another proposed role of the oxidative burst (Bradely et al., 1992; Brisson et al., 1994). ROS may also serve as signal intermediates that induce

phytoalexin biosynthesis (Mehdy, 1994). It is also feasible that ROS play a role in signalling during incompatible interactions. For instance, it has been reported that H_2O_2 strongly induces the genes encoding the defense enzymes, glutathione-S-transferase, glutathione peroxidase, and polyubiquitin (Levine et al., 1994).

Specifically, the $\cdot OH$ radical is an exceptionally strong oxidizing agent which can initiate radical chain reactions with numerous organic molecules. These radical chain reactions include lipid peroxidation and enzyme inactivation (Mehdy, 1994; Lamb and Dixon, 1997). In addition, it has been demonstrated that $\cdot OH$ is capable of fragmenting DNA by site-specific attack (Halliwell and Gutteridge, 1990). Due to its highly reactive nature, $\cdot OH$ is considered the primary ROS responsible for macromolecule modification and cellular damage (Mehdy, 1994). Nevertheless, it has been demonstrated that $O_2^{\cdot -}$ is capable of reacting with proteins containing Fe-S₄ or heme groups, and H_2O_2 is able to react with thiol groups in proteins and reduced glutathione (Thompson et al., 1987). Consequently, the oxidative burst and ROS production can cause significant damage to both host and pathogen cells (Hammond-Kosack and Jones, 1996).

2.2.3 Programmed cell death in plant defense

Another universal feature of the interaction between a plant and an incompatible pathogen is rapid host cell death at the site of pathogen entry. This rapid cell death is termed the HR and it is postulated that a cell death pathway is activated during the HR of plants to invading pathogens. The HR is genetically programmed in the plant and is the result of new transcription and translation. Consequently, plants contain an inherent genetic program that initiates and executes a cell death process on infected cells (Dietrich et al., 1994; Dixon et al., 1994; Godiard et al., 1994; Greenberg et al., 1994; Mittler et al., 1995; Dangl et al., 1996; Jones and Dangl, 1996; Mittler and Lam, 1996).

There are several reports which provide evidence that HR cell death is a form of programmed cell death (PCD) rather than the result of toxins secreted by pathogens that directly kill plant cells. First, active protein synthesis in the plant is essential for

the induction of the HR by pathogenic bacteria (Keen et al., 1981; Croft et al., 1990). Second, it has been established with the use of metabolic inhibitors that the activation of HR cell death by bacterial elicitors called harpins requires active plant metabolism (He et al., 1993). Finally, Zhou et al. (1995) demonstrated that the introduction and overexpression of a *Pti1* transgene isolated from the tomato Pto disease resistance signal transduction pathway, lead to an enhanced HR and cell death response in tobacco to an incompatible bacterium. However, further biochemical and genetic analyses are required to answer the question of whether the HR cell death phenomenon is, in fact, PCD (Jones and Dangl, 1996; Mittler and Lam, 1996).

2.3 THE GENE-FOR-GENE CONCEPT

Studies on the genetics of pathogen virulence during the 1920s to 1940s lead researchers to conclude that virulence (the ability to cause disease) and avirulence (the inability to cause disease) are Mendelian traits and that the segregation of virulence and avirulence alleles could only be tracked on specific host cultivars (Gabriel and Rolfe, 1990). Studies by Flor (1942, 1946, 1971) led to the proposal that these interactions between the host and pathogen may be related to specific interactions between individual host/pathogen genes. Flor established a gene-for-gene relationship between each resistance/susceptibility gene locus in flax (*Linum ultissimum*) and each virulence/avirulence gene locus in the flax rust fungus, *Melampsora lini*. These studies confirmed that avirulence in the rust and resistance in the host are generally determined by single, dominant genes. Therefore, Flor concluded that a host resistance gene was only effective when the microorganism attempting to colonize the host contained a corresponding avirulence gene (Flor, 1942; Flor, 1946; Flor, 1971; Gabriel and Rolfe, 1990; Briggs and Johal, 1994; Crute and Pink, 1996).

Flor's findings have become known as the gene-for-gene model. This model proposes that for resistance to occur, complementary pairs of dominant genes, one in the host (resistance gene) and one in the pathogen (avirulence gene), are required (Hammond-Kosack and Jones, 1997). Therefore, a compatible reaction which results in pathogen colonization due to the lack of an effective host defense response is the

outcome of a host-pathogen interaction unless an allele for resistance at a specific host locus is precisely matched by an allele for avirulence at a specific pathogen locus (Crute and Pink, 1996). Furthermore, a loss or alteration to either the plant resistance (*R*) gene or the pathogen avirulence (*avr*) gene leads to a compatible interaction resulting in disease (Figure 2.1; Hammond-Kosack and Jones, 1997).

Consequently, an *avr* gene gives the pathogen an avirulent phenotype (the inability to cause disease) on a host plant that possesses the corresponding *R* gene, and all other genotypic interactions lead to lack of recognition by the host and the outcome is disease (Staskawicz et al., 1995). Due to this genotypic relationship, these interactions have also been referred to as *R-avr*-dependent incompatibility (Hammond-Kosack and Jones, 1997). Since Flor's discovery of gene-for-gene incompatibility, this relationship has been demonstrated in over 40 different plant/pathogen interactions and holds true for most biotrophic pathogens, including fungi, bacteria, viruses, and nematodes (Gabriel and Rolfe, 1990; Keen, 1990; Crute and Pink, 1996; Hammond-Kosack and Jones, 1997).

The simplest mechanistic interpretation of gene-for-gene interactions is the elicitor-receptor model and this model is also the most compatible with the available genetic and biochemical data (Gabriel and Rolfe, 1990; Keen, 1990; Hammond-Kosack and Jones, 1997). This model theorizes that either primary *avr* gene products, or metabolites resulting from their catalytic activity (elicitors), are recognized by specific plant receptors encoded by the corresponding *R* genes. In addition, it is postulated that this ligand binding stimulates a signalling cascade that eventually leads to the activation of various plant defence responses (Gabriel and Rolfe, 1990; Keen, 1990; Hammond-Kosack and Jones, 1997).

Not all plant disease responses conform to the gene-for-gene model as there is a second plant-pathogen interaction that has different characteristics than those described in Flor's model (Briggs and Johal, 1994; Jones, 1994; Hammond-Kosack and Jones, 1997). This type of interaction involves necrotrophic pathogens and the host-selective toxins and/or enzymes that these pathogens produce (Figure 2.2). For this class of pathogens, plant resistance can be achieved through the loss or alteration of

the toxin or enzyme target site within the plant. In addition, detoxification of the necrotrophic toxin also results in plant resistance (Jones, 1994; Hammond-Kosack and Jones, 1997).

Therefore, this type of interaction differs from the gene-for-gene interaction in that dominant and functional pathogen genes act to cause compatibility rather than incompatibility. Specifically, the necrotrophic compatibility genes control the production of compatibility factors which are molecules that alter host plant physiology to enable colonization by the pathogen (Briggs and Johal, 1994; Hammond-Kosack and Jones, 1997).

Necrotrophic virulence is a dominant trait because of the need to produce functional toxins and/or enzymes, whereas avirulence is a recessive trait (Figure 2.2; Briggs and Johal, 1994; Hammond-Kosack and Jones, 1997). In contrast, in gene-for-gene incompatibility interactions, biotrophic virulence is a recessive trait while avirulence is a dominant trait (Figure 2.1; Gabriel and Rolfe, 1990; Keen, 1990; Crute and Pink, 1996; Hammond-Kosack and Jones, 1997). Accordingly, the type of genetic interaction between plants and necrotrophic pathogens is referred to as toxin-dependent compatibility (Jones, 1994; Hammond-Kosack and Jones, 1997) rather than gene-for-gene incompatibility.

2.4 BACTERIAL AVIRULENCE GENES

2.4.1 Possible roles for *avr* gene products

The only known function of an *avr* gene product in the elicitation of plant disease resistance was discovered by Keen et al. (1990). They reported that expression of the *avrD* gene cloned from *P. syringae* pv. *tomato* leads to the production of a diffusible elicitor which initiates race-specific induction of the HR. The *avrD* gene encodes a cytoplasmic enzyme that converts common bacterial metabolites into low molecular weight *avrD* elicitors known as syringolides (Keen et al., 1990). Since all of the other cloned bacterial *avr* genes encode a diverse group of proteins with few common features and no sequence identity to any known proteins in

the sequence databases, it is unlikely that *avr* gene products besides AvrD possess enzymatic activities (Leach and White, 1996).

Furthermore, the analyses of Avr proteins have not revealed any defining physical characteristics and their sequences do not suggest any biological function except for the *Xanthomonas campestris* pv. *vesicatoria* AvrBs2 protein (described in section 2.10.4 below). These proteins also have no effect when infiltrated into plants and no known biochemical activity except for the *P. syringae* pv. *tomato* AvrD protein (discussed above). Consequently, although over 30 bacterial *avr* genes have been cloned and characterized, the study of the corresponding protein products has yielded minimal information on how they elicit the HR or why bacteria produce them (Alfano and Collmer, 1996; Leach and White, 1996). Therefore, additional studies are required to gain a complete understanding of the function of Avr proteins in eliciting plant resistance.

2.4.2 Role of *avr* genes in pathogenicity

Since the inactivation of *avr* genes in several pathogens does not compromise the pathogenicity of the bacterium and no biochemical or physiological function can be attributed to most *avr* gene products, a common perception has been that *avr* genes play a negligible role in the survival of the pathogen. Thus, it has been considered that most *avr* genes have no selective advantage and, consequently, they are generally believed to be entirely gratuitous (Gabriel and Rolfe, 1990; Leach and White, 1996).

However, mounting evidence suggests *avr* genes may function in other roles besides race-specificity. By comparing phytopathogenic bacteria with mammalian bacterial pathogens, it is possible that Avr proteins are transported directly into plants cells where their intended targets are plant cellular processes involved in resistance (Alfano and Collmer, 1996; Leach and White, 1990). For instance, Kearney and Staskawicz (1990) have established that strains of *Xanthomonas campestris* pv. *campestris* containing an inactivated *avrBs2* gene were less pathogenic on a susceptible cultivar of pepper than *X. campestris* pv. *campestris* containing the wild-type *avrBs2* gene. Thus, mutation of the *avrBs2* locus results in both the loss of

recognition by the *Bs2* resistance gene and diminished bacterial growth in susceptible plants (Kearney and Staskawicz, 1990).

Recently, Swords et al. (1996) also showed that the *avrBs2* locus in *X. campestris* pv. *vesicatoria* is required both for the production of the avirulence signal detected by the *Bs2* resistant host and for full pathogen fitness on susceptible hosts. As well, these researchers ascertained that *X. campestris* pv. *vesicatoria* field isolates that were virulent on pepper plants carrying the *Bs2* resistance gene were specifically disrupted or divergent in the putative AvrBs2 protein. Moreover, these mutants also displayed reduced fitness and it was established that these mutants could be complemented in *trans* by a plasmid-borne copy of *avrBs2* (Swords et al., 1996).

2.4.3 *Avr protein delivery by the Hrp type III protein secretion system*

The activities of all *avr* genes examined to date are dependent upon functional *hrp* (hypersensitivity reaction and pathogenicity) genes. That is, for the *avr* gene products to be recognized by plant cells, the phytopathogenic bacterium must also carry functional *hrp* genes (Huynh et al., 1989; Alfano and Collmer, 1996; Gopalan et al., 1996). Bacterial strains with mutations in *hrp* genes are incapable of eliciting a typical *avr* gene-mediated, race-specific interaction with incompatible host plants (Lindgren et al., 1986; Huynh et al., 1989; Keen et al., 1990; Knoop et al., 1991; Salmeron and Staskawicz, 1993).

Recent studies suggest a Hrp secretory apparatus functions in the secretion of Avr proteins and this Hrp-dependent secretion of Avr proteins is required to trigger the HR in plants during a gene-for-gene incompatible interaction. This assumption is based on the fact that the *hrp* genes of *Erwinia amylovora*, *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria* show sequence similarity to certain genes encoded by the human and animal bacterial pathogenic *Salmonella*, *Shigella*, and *Yersinia* species (Huang et al., 1992; Huang et al., 1993; Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogdanove et al., 1996). These mammalian-cell pathogens encode a type III protein secretion system that delivers bacterial virulence factors through the host cell

membrane directly into the host cell cytoplasm (Rosqvist et al., 1994; Rosqvist et al., 1995; Persson et al., 1995; Barinaga, 1996). Consequently, it is now believed that the *hrp* genes play an integral role in type III protein secretion in phytopathogenic bacteria and that this secretion system delivers Avr proteins directly into plant cells (Alfano and Collmer, 1996; Barinaga, 1996; Gopalan et al., 1996; Leach and White, 1996; Leister et al., 1996a; Pirhonen et al., 1996; Scofield et al., 1996; Swords et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996).

In support of this belief, researchers have recently provided convincing evidence that the Hrp secretory apparatus is involved in Avr signal delivery. Pirhonen et al. (1996) showed that the nonpathogenic *Escherichia coli* elicited a genotype-specific HR in soybean when the *E. coli* were transformed with a cosmid expressing *hrp* genes and a plasmid expressing one of several *avr* genes. This finding established that the expression of the Hrp secretion apparatus is both sufficient for and essential to the delivery of Avr signals (Pirhonen et al., 1996).

Moreover, five other research groups have also provided compelling evidence that the recognition of avirulence proteins occurs inside plant cells and that bacteria may use a Hrp type III secretion system to directly transfer *avr* gene products into plant cells (Gopalan et al., 1996; Leister et al., 1996a; Van den Ackerveken et al., 1996; Scofield et al., 1996; Tang et al., 1996). These research groups all used transient expression assays to determine that the *avrB* (Gopalan et al., 1996; Leister et al., 1996a), *avrRpt2* (Leister et al., 1996a), *avrBs3* (Van den Ackerveken et al., 1996), and *avrPto* (Scofield et al., 1996; Tang et al., 1996) genes elicit resistance responses in a gene-for-gene specific manner only in plants carrying the corresponding resistance genes *RPM1*, *RPS2*, *Bs3*, and *Pto*, respectively.

Therefore, all five research groups (Gopalan et al., 1996; Leister et al., 1996a; Van den Ackerveken et al., 1996; Scofield et al., 1996; Tang et al., 1996) demonstrated that as long as bacterial avirulence proteins are suitably localized within the plant cell they are the only bacterial components required for gene-for-gene interactions. Since the avirulence proteins used by these researchers require the bacterial *hrp* gene cluster to elicit the specific resistance response in plants, it is likely

that AvrB, AvrRpt2, AvrBs3, and AvrPto are translocated directly into the plant cell via the Hrp type III secretion system (Gopalan et al., 1996; Leister et al., 1996a; Van den Ackerveken et al., 1996; Scofield et al., 1996; Tang et al., 1996). Once the Avr proteins are localized inside the plant cells, they either promote parasitism or elicit a genotype-specific HR if the corresponding plant resistance gene is absent or present, respectively (Gopalan et al., 1996).

2.4.4 Concluding remarks

One of the largest obstacles to the study of *avr* genes has been the difficulty of obtaining information on why Avr proteins are produced, how Avr proteins act, and where Avr proteins function (Alfano and Collmer, 1996). However, the recent information obtained by several research groups has helped to answer these questions (Gopalan et al., 1996; Leister et al., 1996a; Scofield et al., 1996; Swords et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996). These new findings have provided valuable insights into the nature, function, and role of bacterial *avr* genes in eliciting genotype-specific hypersensitive cell death and in promoting pathogenic fitness (Leach and White, 1996). Additionally, these experiments verify that the tested bacterial Avr elicitors are recognized within the plant cell and this molecular recognition is consistent with the predicted cytoplasmic location of the protein products of the *RPS2*, *RPM1*, and *Pto* bacterial disease resistance genes (Parker and Coleman, 1997; see section 2.5 below).

These studies have also lent support to the notion that bacterial Avr proteins may be virulence factors that aid pathogen colonization in genetically susceptible plants. As part of an evolving plant surveillance system, it is conceivable that some of these 'virulence factors' have become specifically targeted by R proteins which use this recognition to trigger disease resistance (Parker and Coleman, 1997). Within the near future, scientists will probably learn how each bacterial *avr* gene operates in gene-for-gene resistance and a complete understanding of these *avr* genes will likely lead to improved strategies for disease resistance (Leach and White, 1996).

2.5 PLANT DISEASE RESISTANCE GENE STRUCTURE

2.5.1 Overview

Since 1992, numerous *R* genes from various plant species have been cloned and characterized (Table 2.1). These genes have been shown to confer resistance to diverse bacterial, fungal, viral, and nematode pathogens and, remarkably, their gene products share striking structural similarities. This homology suggests that certain signalling events in the plant defense response are shared among various plant species. Currently, there are five classes of *R* genes (Baker et al., 1997; Table 2.1). Only one class of these *R* genes is involved in toxin-dependent compatibility while the other four classes of *R* genes are involved in gene-for-gene incompatibility.

2.5.2 Class 1 - Cytoplasmic protein kinase *R* genes

The first class of *R* genes encode cytoplasmic serine/threonine protein kinases. Currently, the *Pto* gene is the only gene belonging to this class. In tomato, the *Pto* gene confers resistance to strains of *P. syringae* pv. *tomato* that express the avirulence gene *avrPto* (Ronald et al., 1992; Martin et al., 1993a). The *Pto* kinase is the only product of an *R* gene participating in a gene-for-gene interaction that does not possess leucine-rich repeats (LRRs). The remaining *R* genes are categorized by the presence of LRRs and other structural motifs in their protein products.

2.5.3 Class 2 - Cytoplasmic NBS-LRR *R* genes

The second class of *R* genes encode cytoplasmic receptor-like proteins that contain a leucine-rich repeat (LRR) domain and an ATP/GTP nucleotide binding site (NBS). The family of genes that encodes proteins with the LRR and NBS structural motifs includes *RPS2*, *RPM1*, and *RPP5* from *Arabidopsis*; *L6* and *M* from flax; *N* from tobacco; and *Prf* and *I2C-1* from tomato. This class of *R* genes can be further subdivided into two distinct groups based on the presence of additional structural motifs (Table 2.1; Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997; Ori et al., 1997).

RPS2 is a resistance gene of *Arabidopsis thaliana* that confers resistance against isolates of the bacterial pathogen, *P. syringae*, that express the avirulence gene, *avrRpt2* (Bent et al., 1994; Mindrinos et al., 1994). *RPM1* is another *A. thaliana* *R* gene that confers resistance against *P. syringae*. However, unlike *RPS2*, the *RPM1* gene conditions resistance to *P. syringae* isolates that express either of the two unrelated avirulence genes, *avrRPM1* or *avrB* (Grant et al., 1995). Recently, Leister et al. (1996a) provided indirect evidence that indicated *RPS2* is cytoplasmically localized and, due to the close similarity between *RPS2* and *RPM1*, these authors suggested that *RPM1* is also cytoplasmically localized.

The *I2C-1* gene genetically confers disease resistance to *Fusarium oxysporum* forma specialis (f. sp.) *lycopersici* race 2 in a gene-for-gene manner (Ori et al., 1997). Mutagenesis of tomato lines carrying *Pto* revealed that an additional gene, *Prf*, is required for *Pto* to function (Salmeron et al., 1994). Interestingly, *Prf* encodes a protein that falls into the *RPS2*, *RPM1*, and *I2C-1* class of resistance gene products.

In addition to possessing LRR and NBS structural motifs, the *RPS2*, *RPM1*, *Prf* and *I2C-1* proteins also contain an additional leucine zipper (LZ) motif (Table 2.1; Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997; Ori et al., 1997). However, the leucine zippers (LZs) of *RPS2*, *RPM1*, and *Prf* are located at their amino-termini (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Salmeron et al., 1996) while the LZ of *I2C-1* is found in its carboxy-terminus (Ori et al., 1997).

N, *L6*, *M*, and *RPP5* form the second subgroup of the NBS-LRR *R* genes since they encode putative proteins that have large amino-terminal domains with homology to cytoplasmic domains of the *Drosophila* Toll protein and the mammalian interleukin-1 receptors (IL-1R; Table 2.1). As a result, this amino-terminal domain of these *R* genes has been referred to as the TIR domain for Toll-IL-1R homology region (Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997).

The tobacco *N* gene mediates resistance to the viral pathogen, tobacco mosaic virus (TMV; Whitham et al., 1994). No transmembrane domain or signal sequence was discovered in the *N* product and, consequently, the authors suggested that it is a cytoplasmic protein (Whitham et al., 1994). The flax *L6* gene confers resistance to

different isolates of the flax rust fungus, *Melampsora lini* (Lawrence et al., 1995). It was established that the amino-terminus of the L6 protein also contains a potential signal peptide but no additional extended hydrophobic regions that could provide a membrane-spanning domain (Lawrence et al., 1995).

M is also a flax disease resistance gene that confers resistance to the rust fungal pathogen, *M. lini* (Anderson et al., 1997). *M* is closely related to the unlinked *L6* gene and, like *L6*, *M* is predicted to encode a product of the NBS-LRR-TIR class of disease resistance proteins. Translation of the open reading frame of the *M* gene predicted a polypeptide product that is 78% identical and 85% similar to *L6* and contains all of the motifs identified in *L6* (Anderson et al., 1997). Recently, the *Arabidopsis RPP5* gene specifying resistance to the biotrophic downy mildew fungus, *Peronospora parasitica*, was positionally cloned (Parker et al., 1997). The *RPP5* sequence was examined for a potential signal sequence and for membrane-spanning regions but both were absent from the protein. Consequently, the authors suggested that *RPP5* is a cytoplasmic protein (Parker et al., 1997).

2.5.4 Class 3 - LRR-transmembrane *R* genes

The third class of *R* genes currently includes three genes (Table 2.1). The common motifs shared between the predicted products of these genes are a LRR region and a transmembrane domain. The first gene to be cloned from this class was the tomato *Cf-9* gene which confers resistance to infection by races of the fungus, *Cladosporium fulvum*, that express the avirulence gene *Avr9* (Jones et al., 1994). The *Cf-9* gene appears to encode a putative transmembrane receptor. Accordingly, *Cf-9* likely encodes an extracytoplasmic glycoprotein anchored to a cell membrane, with the majority of the extracytoplasmic domain made of LRRs (Jones et al., 1994).

The tomato *Cf-2* locus was shown to comprise two almost identical genes which can independently confer resistance to isolates of the tomato leaf mold, *C. fulvum*, that express the corresponding *Avr2* gene (Dixon et al., 1996). Following the sequencing of these genes it was established that only three nucleotides differ between the open reading frames and each of these nucleotide substitutions causes an amino

acid substitution. The Cf-2 proteins show greatest homology to Cf-9 and can be subdivided into the same functional domains (Dixon et al., 1996).

The sugar beet *HsI^{pro-1}* gene was recently isolated and also encodes a LRR-transmembrane protein that confers resistance to the beet cyst nematode, *Heterodera schachtii* Schmidt (Cai et al., 1997). No sequences with homology to the *HsI^{pro-1}* nucleotide sequence or *HsI^{pro-1}* amino acid sequence were detected after comparing these sequences to known genes and proteins in the current databases (Cai et al., 1997). However, the *HsI^{pro-1}* protein contains functional domains corresponding to the Cf-9 and Cf-2 proteins. As a result, although no significant sequence homology was detected between *HsI^{pro-1}* and Cf-9 or Cf-2, similar protein structures between these proteins exist (Cai et al., 1997). Therefore, *HsI^{pro-1}* falls into the LRR-transmembrane class of *R* genes (Baker et al., 1997; Cai et al., 1997).

2.5.5 Class 4 - LRR-transmembrane protein kinase *R* genes

The fourth class of *R* genes encode putative transmembrane receptors that possess extracellular domains and intracellular kinase domains (Table 2.1). At present, the *Xa21* gene (Song et al., 1995) from rice is the only member of this class. Moreover, *Xa21* is the only *R* gene participating in a gene-for-gene interaction to be cloned from a monocotyledonous plant.

The rice *Xa21* gene confers resistance to the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* race 6 (Song et al., 1995). The amino-terminus of the *Xa21* protein contains a potential signal peptide. The protein also encodes an extracellular LRR region and a possible membrane-spanning helix. Finally, the *Xa21* protein also possesses a putative intracellular serine/threonine kinase catalytic domain. Consequently, the sequence of the predicted protein indicates that it is a receptor-like kinase (Song et al., 1995).

2.5.6 Class 5 - Toxin reductase *R* genes

The first plant *R* gene to be cloned was the maize *HMI* gene that provides resistance against toxin-dependent compatibility (Table 2.1; Johal and Briggs, 1992).

In previous studies, it was ascertained that, in maize, specific resistance to the pathogenic fungus *Cochliobolus carbonum* race 1 is determined by the *Hm* gene (Nelson and Ullstrup, 1964). Several studies showed that the specific pathogenicity of *C. carbonum* is mediated by a low molecular weight compound called HC-toxin and that *Hm* directs the maize defense response by making the plant insensitive to HC-toxin (Yoder, 1980).

Following these studies, it was demonstrated that an enzyme from maize, called HC-toxin reductase (HCTR), inactivated the HC-toxin (Meeley and Walton, 1991) and this inactivation of the HC-toxin is the biochemical basis of *Hm*-specific resistance in maize to infection by *C. carbonum* race 1 (Meeley et al., 1992). In 1992, the *HMI* gene was cloned and it was shown to control both race-specific resistance to race 1 isolates of *C. carbonum* and expression of HCTR (Johal and Briggs, 1992). Sequence homology with dihydrofavonol-4-reductase genes demonstrated that *HMI* encodes a NADPH-dependent reductase (Johal and Briggs, 1992).

It must be noted that the studies of *HMI* did not suggest a structure or function for *R* genes involved in gene-for-gene incompatibility since the toxin-detoxifying method of *HMI*-resistance does not involve pathogen *Avr* genes, elicitation of hypersensitive cell death, or other aspects of gene-for-gene interactions (Bent, 1996). Additionally, the genetics of the interaction between maize and *C. carbonum* do not conform to the gene-for-gene model of incompatibility since toxin-deficient *C. carbonum* races lose their ability to cause disease in maize cultivars that do not carry *HMI* (Staskawicz et al., 1995).

2.6 R PROTEIN MOTIFS AND THEIR POTENTIAL FUNCTION

2.6.1 Pathogen recognition

Since the cloning and characterization of *R* genes has revealed that numerous *R* gene products possess structural and functional domains characteristic of actual receptor proteins found in *Drosophila*, mammals, and yeast, it is reasonable to assume

that plant R proteins have a receptor-like function. The likely candidate motif for providing the recognition specificity and ligand binding is the LRR domain (Baker et al., 1997; Hammond-Kosack and Jones, 1997) since LRRs are known to be involved in protein-protein interactions and ligand binding in proteins participating in signal transduction pathways in eukaryotes (Kobe and Deisenhofer, 1994).

For instance, the amino-terminal, extracytoplasmic LRRs of Cf-9, Cf-2, and Hs1^{pro-1} may function as receptors for recognizing putative elicitors (Jones et al., 1994; Dixon et al., 1996; Cai et al., 1997) since the Cf-9, Cf-2, and Hs1^{pro-1} proteins all have extracellular domains that would be well positioned to encounter the intercellular pathogens, *C. fulvum* and *H. schachtii* (Parker and Coleman, 1997). Another scenario is that the LRRs of these proteins serve as domains for interactions with other membrane-bound, plant proteins that bind the elicitors (Jones et al., 1994; Dixon et al., 1996). The possibility that extracellular LRRs may function in the binding of pathogen-produced elicitors could also apply to the rice Xa21 protein if the unidentified *avrXa21* gene product is secreted from bacteria but does not enter the plant cells (Hammond-Kosack and Jones, 1997).

The possible role of LRR domains in binding *avr* determinants is not restricted to transmembrane proteins that possess extracellular LRRs. The direct interaction between the predicted cytoplasmic N protein and the TMV replicase product is likely to occur (Whitham et al., 1994) because TMV replication is strictly intracellular (Padgett and Beachy, 1993). Consequently, it is also possible that the LRR domain of N participates in directly binding the viral *avr* determinant. And, as described previously, it is now believed that the *Avr* gene products of bacterial pathogens are directly transferred into plant cells via the Hrp type III secretion pathway (Alfano and Collmer, 1996; Barinaga, 1996; Gopalan et al., 1996; Leach and White, 1996; Leister et al., 1996a; Pirhonen et al., 1996; Scofield et al., 1996; Swords et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996). As a result, the LRR domains of the cytoplasmically located RPS2, RPM1, and Prf proteins could also be conceivably involved in directly binding *avr* gene products.

Therefore, only the putative cytoplasmic L6, M, RPP5, and I2C-1 fungal

resistance proteins have LRR domains that do not seem to have any role in a *avr* gene product recognition. The LRR domains of these proteins may not participate in *avr* determinant recognition since the transport of fungal elicitor molecules into plant cells is unknown and, consequently, how these proteins recognize the corresponding fungal elicitors remains obscure. Nevertheless, it is possible that fungi could inject Avr proteins into the cytoplasm of a plant cell through haustoria (Parker and Coleman, 1997) and, as a result, the LRRs of L6, M, RPP5, and I2C-1 may also function in binding *avr* determinants.

2.6.2 Signal transduction

After the pathogen has been recognized in an incompatible interaction, it is predicted that *R* genes activate signalling cascade(s) which coordinate the initial plant defense responses that impede colonization by the pathogen. But, at present, there is insufficient data to determine the function of the different *R* protein domains in the resistance signalling pathway. Nevertheless, the LZ, NBS, TIR, and kinase domains of *R* gene products are found in numerous eukaryotic proteins participating in various signal transduction pathways and preliminary data indicate that these domains are essential for *R* gene function (Baker et al., 1997).

The LZ regions of RPS2, RPM1, Prf, and I2C-1 could potentially facilitate the homodimerization of the proteins themselves or heterodimerization with other proteins involved in the signalling cascade (Bent, 1996; Hammond-Kosack and Jones, 1997). The LZ could promote the dimerization of transcription factors by formation of a coiled-coil structure (Bent et al., 1994; Bent, 1996).

Numerous *R* genes also encode proteins with potential nucleotide binding sites (NBSs). These NBS domains are found in diverse families of proteins with ATP and GTP binding activity such as the RAS group, adenosine triphosphatases (ATPases), adenylate kinases, heterotrimeric GTP-binding proteins (G proteins), and ribosomal elongation factors (Saraste et al., 1990; Traut, 1994). Since all of the cytoplasmically located LRR-containing *R* proteins possess amino-terminal, highly conserved NBS domains, it is likely that nucleotide triphosphate binding is essential in order for these

proteins to function in the signalling cascade (Bent, 1996; Hammond-Kosack and Jones, 1997; Baker et al., 1997). The fact that a nonfunctional, *prf* mutant carries an amino acid change which eliminates a residue conserved in the nucleotide binding domain (Salmeron et al., 1996) supports the requirement of functional NBS domains for R protein biological activity.

It is also likely that the TIR domains found in N, L6, M, and RPP5 play a role in the signal transduction cascade of the resistance pathway. Recently, it has been established that the *Drosophila* Toll protein may be involved in the nuclear localization of a Rel-related transcription factor called Dif (Rosetto et al., 1995). Several studies have determined that Toll and Dif play a role in the immune response of *Drosophila* adults to pathogen invasion (Ip et al., 1993; Rosetto et al., 1995) and this pathway results in the activation of antimicrobial host responses (Ip et al., 1993; Lemaitre et al., 1995; Lemaitre et al., 1996). In the mammalian IL-1 pathway, interaction of IL-1R with the ligand IL-1 results in the activation of the Rel-family transcription factor, NF- κ B. The activated NF- κ B protein is then translocated to the nucleus where it induces the synthesis of a variety of defense and signalling proteins involved in inflammatory, immune, and acute phase responses (Baeuerle, 1991; Wasserman, 1993; Baker et al., 1997; Hammond-Kosack and Jones, 1997).

Since N, L6, M, and RPP5 all possess large amino-terminal domains with homology to the cytoplasmic signalling domains of the *Drosophila* Toll and mammalian IL-1R proteins, it is reasonable to assume that these plant R proteins activate an intracellular signal transduction cascade related to the Toll and IL-1R pathways and this potential cascade likely induces plant genes responsible for the incompatible reaction. Consequently, N, L6, M, and RPP5 would also have to function in an analogous manner to Toll and IL-1R. In essence, the downstream effector capability of these *R* gene-encoded proteins would be governed by their conserved, amino-terminal, TIR domains (Whitham et al., 1994; Baker et al., 1997; Hammond-Kosack and Jones, 1997; Parker et al., 1997).

The serine/threonine kinase domains of Pto and Xa21 have an obvious role in signal transduction during the defence response since the alteration of the

phosphorylation state of proteins is a routine mechanism that organisms use to regulate protein activity. Because the Pto kinase shares homology with the *Drosophila* pelle and mammalian IRAK serine/threonine kinases (Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997) it is possible that Pto, like pelle and IRAK, acts as a transcription factor activator.

Unlike Pto, the Xa21 kinase possesses both a putative extracellular LRR motif and cytoplasmic kinase domain. The extracellular domain is 55% similar to Cf-9 and the serine/threonine kinase domain is 57% similar to Pto (Song et al., 1995). These strong similarities indicate that signalling through the LRR-transmembrane *R* gene-encoded proteins (Cf-like proteins), which lack any obvious signal transmission domains, may occur through a serine/threonine kinase. One possibility is that Cf-like and Pto-like proteins may constitute a two-component receptor system resembling the transmembrane LRR kinase, Xa21. The short, cytoplasmic domains of the Cf-like proteins could interact with a Pto-like kinase to permit activation of downstream components of the signalling cascade. Another possibility is that Cf-like proteins may already exist or become associated with a membrane receptor complex involving a LRR transmembrane kinase analogous to Xa21. Both of these scenarios would permit Cf-like proteins to recruit a kinase domain for intracellular signalling (Bent, 1996; Boyes et al., 1996; Dixon et al., 1996; Baker et al., 1997; Hammond-Kosack, 1997).

2.7 FUTURE PROSPECTS

As more *R* genes are cloned and characterized, knowledge will be gained on the *R* protein structural requirements for Avr protein recognition and defence response initiation. Analyses of closely related *R* proteins will also permit scientists to elucidate the function and location of recognition and signalling domains. Finally, it is also possible that the numerous, currently cloned *avr* genes can be used as baits in the two-hybrid system to isolate their *R* gene counterparts (Lamb, 1996; Parker and Coleman, 1997).

In the near future, valuable information will be gained from structure-function and ligand-receptor studies. Additionally, data will also be obtained from the genetic

dissection of *R* gene mediated induction of the defense response. All of this information will then be employed to engineer crops that will be resistant to a broad range of pathogens. For instance, access to cloned *R* genes will allow plant breeders to improve plant breeding strategies by using marker assisted selection and plant transformation. These new strategies will accelerate *R* gene introgression into agronomically important crops that are susceptible to prevalent pathogens (Baker et al., 1997; Hammond-Kosack and Jones, 1997).

2.8 THE *Pto*-MEDIATED SIGNAL TRANSDUCTION PATHWAY

Pto was the first *R* gene participating in a gene-for-gene interaction to be cloned (Martin et al., 1993a). *Pto* is a wild tomato (*Lycopersicon pimpinellifolium*) gene that confers resistance to bacterial speck disease caused by strains of *P. syringae* pv. *tomato* which possess the avirulence gene, *avrPto* (Ronald et al., 1992). Tomato carrying the *Pto* locus is a model system for the study of signal transduction pathways mediated by a specific *R* gene. This system is the only example of an *R* gene-mediated resistance pathway from which numerous genes encoding multiple components have been cloned and from which all of the known gene products have been characterized (Martin et al., 1993a; Zhou et al., 1995; Salmeron et al., 1996; Zhou et al., 1997).

A map-based cloning approach was used to isolate a complementary DNA (cDNA) clone that represented a gene family containing at least six members that cosegregated with *Pto* (Martin et al., 1993a). Using segregation analysis and inoculation with *P. syringae* pv. *tomato*, it was determined that a cDNA (CD186) functionally complemented *pto* in susceptible plants by inhibiting the growth of the bacterial population and suppressing symptoms of bacterial speck disease (Martin et al., 1993a).

The entire 2.4 kilobase pair (kbp) DNA sequence of CD186 was determined and a 963 base pair (bp) open reading frame (ORF) was located. The ORF was named *Pto* and encoded a 321 amino acid hydrophilic serine/threonine protein kinase. It was also shown that the *Pto* protein does not contain a membrane-spanning or

extracellular domain but does contain a potential myristoylation site at the amino terminus. Therefore, the authors suggested that association with the plasma membrane could place *Pto* in close proximity to an extracellular receptor having a cytoplasmic domain. It was also postulated that *Pto* may exist in the cytoplasm and participate at a later step of a phosphorylation cascade (Martin et al., 1993a).

Two years after cloning *Pto*, it was determined that the *Pto* protein was capable of autophosphorylation *in vitro* (Loh and Martin, 1995a; Loh and Martin, 1995b; Rommens et al., 1995a). Loh and Martin (1995b) also established that the *Pto* kinase phosphorylates serine and threonine residues but not tyrosine residues. Consequently, these results suggested that *Pto* is a serine/threonine kinase that participates in a phosphorylation cascade which eventually leads to resistance against the bacterial pathogen, *P. syringae* pv. *tomato* carrying the *avrPto* avirulence gene (Loh and Martin, 1995b).

Until 1996, the molecular basis of the gene-for-gene model was unknown even though the available genetic and biochemical data lent support to the elicitor-receptor model (Gabriel and Rolfe, 1990; Keen, 1990; Hammond-Kosack and Jones, 1997). This elicitor-receptor model was finally proven two years ago. In two independent studies, Scofield et al. (1996) and Tang et al. (1996) employed the yeast two-hybrid system to provide decisive evidence that there is a direct interaction between the bacterial *avrPto* gene product and the tomato *Pto* resistance gene product. The physical interaction of *AvrPto* with *Pto* implied that *AvrPto* functions as a bacterial signal molecule and *Pto* functions as the corresponding plant receptor. Moreover, this finding demonstrates that *Pto*-mediated disease resistance involves entry of *avrPto* into the plant cell cytoplasm (Scofield et al., 1996; Tang et al., 1996).

The relevance of the *Pto*-*AvrPto* interaction was then tested by examining inactive alleles of *avrPto* and *Pto*. Scofield et al. (1996) established that the protein products of three inactive, mutant *Pto* alleles showed no detectable interaction with *AvrPto* in the yeast two-hybrid system and, thus, mutant *Pto* alleles that confer susceptibility to *P. syringae* pv. *tomato* (*avrPto*) also fail to interact with *AvrPto*. Several carboxy-terminal deletions of *AvrPto* were also assayed for interaction with

Pto in the two-hybrid system (Scofield et al., 1996; Tang et al., 1996).

Deletion of up to 30 amino acids from the carboxy-terminus of AvrPto did not prevent AvrPto from interacting with Pto. However, the deletion of 41 or more amino acids from the carboxy-terminus of AvrPto abolished the interaction of AvrPto with Pto. The *avrPto* carboxy-terminal deletions were introduced into *P. syringae* and these bacteria were inoculated onto plants expressing *Pto*. Only the deletions that interacted with Pto in the two-hybrid system induced both an HR and disease resistance. Consequently, mutations of *Pto* or *avrPto* that disrupt Pto-avrPto interactions in the two-hybrid system also abolish disease resistance in plants (Scofield et al., 1996; Tang et al., 1996).

As will be discussed below, *Pto* is a member of a tightly linked gene family containing the *Fen* gene which encodes a kinase with 80% identity and 87% similarity to the Pto kinase. However, *Fen* does not confer *Pto*-like resistance but, instead, confers sensitivity to the insecticide, fenthion (Martin et al., 1994; see below). Since *Fen* does not interact with *avrPto*, it was used in domain-swap experiments to determine if particular regions of Pto are required for interaction with AvrPto (Scofield et al., 1996; Tang et al., 1996).

Using Pto-Fen chimeric proteins and the yeast two-hybrid system, Tang et al. (1996) determined that a region in Pto from amino acids 129 to 224 is required for interaction with AvrPto. In analogous experiments using the two-hybrid system but different Pto-Fen hybrid constructs, Scofield et al. (1996) also demonstrated that the carboxy-terminal 192 amino acids of Pto contain the residues necessary to confer binding to AvrPto. However, they specifically identified residues 190 to 213, rather than residues 129 to 224, as being critical for specific AvrPto binding (Scofield et al., 1996).

To elucidate the role of Pto kinase function in the interaction with AvrPto, two invariant residues essential for kinase activity in Pto were mutated and these two mutants were assayed for AvrPto-binding activity in the two-hybrid system. Neither kinase mutant showed detectable interaction with AvrPto, indicating that the phosphorylation activity of Pto is required for the binding of AvrPto (Scofield et al.,

1996).

It has also been ascertained that the *Pto* kinase mediates a signalling pathway leading to the oxidative burst in tomato (Chandra et al., 1996). Using *P. syringae* pv. *tomato* ($\pm$ *avrPto*), it was established that the oxidative burst is dependent on coexpression of *Pto* in tomato and *avrPto* in *P. syringae* pv. *tomato*. In the other three plant-pathogen interactions, the authors either did not observe an oxidative burst or they observed only the phase one burst (Chandra et al., 1996).

A significant advancement in the engineering of plants for improved disease resistance occurred when *Pto* disease resistance was transferred to tobacco (Rommens et al., 1995b; Thilmony et al., 1995). These two research groups independently provided evidence that transformation of tobacco with *Pto* results in resistance to *Pseudomonas syringae* pv. *tabaci* strains expressing *avrPto*. Specifically, they showed that the *Pto*-transformed tobacco plants exhibited a significant increase in resistance to *P. syringae* pv. *tabaci* (*avrPto*) when compared to wild-type tobacco. This enhanced resistance was marked by the development of the HR and inhibition of avirulent *P. syringae* pv. *tabaci* (*avrPto*) growth in inoculated leaves (Rommens et al., 1995b; Thilmony et al., 1995). Rommens et al. (1995b) also determined that *Pto*-resistance cosegregated with *Pto* during transgene selfings and testcrosses (Rommens et al., 1995b). Consequently, both research groups suggested that components of a *Pto*-mediated signal transduction pathway are conserved in tobacco and the utility of host-specific resistance genes can be extended by intergenic transfer (Thilmony et al., 1995; Rommens et al., 1995b).

Another unique feature of the *Pto*-*avrPto* interaction is that a parallel signalling pathway exists. This pathway is activated by the organophosphorous insecticide, fenthion, and causes the formation of small, necrotic lesions in tomato plants that contain the *Pto* locus. These lesions resemble the lesions produced by hypersensitive cell death in response to avirulent bacteria (Carland and Staskawicz, 1993; Martin et al., 1993b; Martin et al., 1994).

Analysis of the *Pto* locus indicated that this gene is part of a locus consisting of five to eight genes clustered within a 400 kbp region on chromosome 5 that are all

homologous to *Pto* (Martin et al., 1993a; Martin et al., 1994). One of these *Pto* homologs is *Fen* and it specifies sensitivity to fenthion (Martin et al., 1994). Confirmation that the *Fen* gene is separate from the tightly linked *Pto* gene was revealed by mutational analysis (Salmeron et al., 1994) and by ascertaining that the isolated *Fen* gene confers sensitivity to fenthion but not to *P. syringae* pv. *tomato* carrying *avrPto* in stable transformants (Martin et al., 1994).

The *Fen* gene codes for a 318 amino acid serine/threonine protein kinase that shares 80% identity and 87% similarity with the *Pto* protein. As with *Pto*, the *Fen* protein is comprised primarily of a protein kinase catalytic domain with no apparent extracellular or transmembrane domain. In addition, both *Fen* and *Pto* proteins are identical over a 15 amino acid region containing a potential myristoylation site (Martin et al., 1994). The *Fen* protein is also a functional kinase that is able to autophosphorylate *in vitro* (Loh and Martin, 1995a; Loh and Martin, 1995b; Rommens et al., 1995a).

Mutation of the putative N-terminal myristoylation site rendered the *Fen* protein inactive and analysis of a *Pto-Fen* chimeric gene allowed the fenthion sensitivity domain to be localized to the C-terminal region of the *Fen* protein. Additionally, the expression of the *Fen* kinase in tobacco resulted in a fenthion-dependent necrotic response (Rommens et al., 1995a). Loh and Martin (1995b) also established that the *Fen* kinase phosphorylates serine and threonine residues but not tyrosine residues. In addition, *Fen* kinase activity is eliminated when the nucleotides that encode a conserved lysine residue at an ATP binding site in subdomain II are mutated (Loh and Martin, 1995b). These results lead the authors to conclude that the *Fen* protein is a functional serine/threonine kinase that is involved in a signal transduction pathway(s) that leads to fenthion sensitivity (Loh and Martin, 1995b; Rommens et al., 1995a).

Recently, the yeast two-hybrid system was used to identify *Pti1* (*Pto*-interacting 1), a second serine/threonine protein kinase that participates in the *avrPto*-specific defense response by physically interacting with *Pto* (Zhou et al., 1995). Like *Pto* and *Fen*, *Pti1* does not possess a transmembrane or extracellular domain but, unlike *Pto*

and Fen, Pti1 lacks a putative myristoylation site. Based on these observations, Zhou et al. (1995) suggested that Pti1 is probably a cytoplasmic protein kinase.

The authors used fusion protein expression studies to demonstrate that Pti1 is capable of autophosphorylation but incapable of phosphorylating Pto. Using similar experiments, Pto was shown to phosphorylate Pti1 and this phosphorylation occurred exclusively on serine and threonine residues of Pti1. In addition, it was established that a kinase-deficient Pto protein was unable to physically interact with Pti1 and expression of a *Pti1* transgene in tobacco plants enhanced the HR to a *P. syringae* pv. *tabaci* strain carrying *avrPto*. These results suggest that Pti1 is involved in the Pto-mediated signalling pathway by acting downstream of Pto in a protein kinase cascade that eventually leads to the development of the HR and contributes to overall disease resistance (Zhou et al., 1995).

In additional experiments, no phosphorylation was observed between Fen and Pti1 (Zhou et al., 1995). The specific phosphorylation of Pti1 by Pto but not by Fen strongly implies that Pti1 is part of a kinase cascade that is specific to *avrPto-Pto* signalling. Thus, a different component must act downstream of Fen and, perhaps, a homolog of Pti1 serves as the specific substrate for Fen (Zhou et al., 1995).

Very recently, researchers used the two-hybrid system to identify three distinct genes, *Pti4*, *Pti5*, and *Pti6*, that encode proteins that also specifically interact with the Pto kinase in the yeast two-hybrid system (Zhou et al., 1997). These genes all encode proteins with characteristics that are typical of transcription factors and are homologous to the tobacco ethylene-responsive element-binding proteins (EREBPs). *Pti4*, *Pti5*, and *Pti6* all possess a highly conserved DNA-binding domain and this domain was shown to be involved in the interaction with Pto. Additionally, it was established that these transcription factors are functionally similar to EREBPs in both structure and DNA binding properties. Finally, it was demonstrated that *Pti4*, *Pti5*, and *Pti6* specifically recognize and bind to a DNA sequence that is present in the promoter region of numerous genes encoding PR proteins (Zhou et al., 1997).

The authors suggest that *Pti4*, *Pti5*, and *Pti6* are involved in defense gene regulation. This possibility is supported by the fact that EREBP-2 and Pto strongly

interact; EREBP-1 is induced early upon *Pto-avrPto* recognition; and EREBP-1 is induced prior to several endogenous PR genes in the incompatible interaction. Furthermore, expression of several PR proteins is specifically enhanced upon *Pto-avrPto* recognition. Therefore, Pti4, Pti5, Pti6 and at least a few of the EREBPs are conceivably involved in linking an *R* gene product (*Pto*) to the expression of defense-related genes (Zhou et al., 1997). Although the role of PR proteins in plant defense has yet to be determined, these results establish, for the first time, a sequence of events that link a specific Avr-R protein interaction with defence-related gene activation (Parker and Coleman, 1997; Zhou et al., 1997).

Another gene has also been identified in both *Pto* resistance and fenthion sensitivity (Salmeron et al., 1994; Salmeron et al., 1996). This locus was designated *Prf* (for *Pseudomonas* resistance and fenthion sensitivity) and was shown to play a role in tomato signalling in response to both pathogen elicitors and fenthion. Specifically, this gene was identified by the isolation of tomato mutants that were both susceptible to *P. syringae* pv. *tomato* (*avrPto*) and insensitive to fenthion. Since it was established that *Prf* is required for both *avrPto*-specific resistance and fenthion sensitivity, the authors suggested that *Prf* likely encodes a second component of the tomato *Pto* disease resistance and fenthion sensitivity pathways. Genetic analysis of F_2 populations from crosses of *pto* and *prf* homozygotes also demonstrated that the *Pto* and *Prf* loci are tightly linked (Salmeron et al., 1994). *Prf* was later shown to be located in the middle of the *Pto* gene cluster, 24 kbp from the *Pto* gene and 500 bp from the *Fen* gene (Salmeron et al., 1996).

Recently, alleles of *Pto* and *Fen* have been isolated and characterized from cultivated tomato, *Lycopersicon esculentum* (Jia et al., 1997). These alleles were designated *pto* and *fen* and have uninterrupted open reading frames (ORFs) that code for predicted proteins that are 87% and 98% identical to the *Pto* and *Fen* protein kinases, respectively. Both the *pto* and *fen* alleles were shown to encode active kinases and, like *Pto* and *Fen*, the *pto* kinase phosphorylates the Pti1 kinase but the *fen* kinase does not (Jia et al., 1997).

However, the protein encoded by the *pto* allele is impaired in its ability to physically interact with Pti1 and several other Pto-interacting proteins. Since *pto* and *fen* are active kinases but are incapable of conferring disease resistance or fenthion sensitivity, the authors proposed that the differences in the deduced amino acids between *pto* and Pto, and *fen* and Fen generate hindrances in signal molecule recognition or protein-protein interactions involved in the *Pto*- and *Fen*-mediated signal transduction pathways (Jia et al., 1997). In support of this assumption, it has also been established that *pto* does not interact with Pti4, Pti5, or Pti6, confirming that tomato plants carrying *pto* are susceptible to *P. syringae* pv. *tomato* expressing *avrPto* (Zhou et al., 1997).

2.9 BACTERIA AS PLANT PATHOGENS

Not only are we consumers of plants, but plants can serve as an excellent source of nutrients for numerous bacterial species. Most bacteria readily enter the apoplast of plants through stomates and other natural openings. Nonetheless, relatively few bacteria can successfully infect plants presumably because the process requires metabolic intimacy between a specialized pathogen and host plant, a condition that does not frequently occur (Agrios, 1988; Alfano and Collmer, 1996).

Although few bacterial species are plant-pathogenic (phytopathogenic), those that cause many serious plant diseases. Infected plants exhibit a wide range of disease symptoms and each type of symptom can be caused by several genera of phytopathogenic bacteria. Furthermore, within each genus there are individual bacterial isolates which cause several different plant diseases (Agrios, 1988; Jackson and Taylor, 1996).

Approximately, 80 species of bacteria have been shown to cause infectious diseases in plants. Many of these 80 species are taxonomically subdivided into several pathovars or strains that apparently differ only in the plant species they infect. All of these phytopathogenic bacteria can further be divided based on host-pathogen interactions, of which the majority of bacterial species are facultative saprophytes (Agrios, 1988).

The gram-negative, rod-shaped, bacteria of the genera *Erwinia*, *Pseudomonas*, and *Xanthomonas* are the most prevalent and destructive phytopathogenic bacteria (Jackson and Taylor, 1996). The members of these genera are capable of causing necrosis, that is, localized cell death and degeneration of tissues. In addition, their necrogenic ability is divided into two distinct categories, one group utilizing biotrophic pathogenesis and the other group utilizing necrotrophic pathogenesis (Alfano and Collmer, 1996; Collmer and Bauer, 1994; Dickinson and Lucas, 1982).

Biotrophic pathogens normally multiply within the host tissue for a prolonged period prior to causing necrosis and killing the plant. This mechanism of pathogenesis allows for optimum pathogen replication without killing the host plant immediately. Biotrophs use this process because they are dependent upon viable host tissue to complete their development. Consequently, biotrophs have a specialized form of parasitism which limits their host range (Alfano and Collmer, 1996; Collmer and Bauer, 1994; Dickinson and Lucas, 1982; Jackson and Taylor, 1996).

In contrast, the necrotrophic pathogens utilize a fierce infection process that results in the rapid killing of parenchymatous tissues during active pathogenesis. The necrotrophs are often opportunistic pathogens which invade wounds and juvenile or weakened plant tissues. They grow intercellularly within the host and produce cytolytic factors. These factors lyse the host cells and then the necrotrophs use the dead host tissues as an energy source. Consequently, necrotrophic pathogens kill part or all of their hosts before deriving nutrients from them while biotrophic pathogens derive nutrients from the living tissues of their hosts (Alfano and Collmer, 1996; Collmer and Bauer, 1994; Dickinson and Lucas, 1982; Jackson and Taylor, 1996).

2.10 THE DISEASE CYCLE OF BACTERIAL PHYTOPATHOGENS

The sequence of events occurring between a pathogenic bacterium and a host plant in relation to disease development is known as the disease cycle. This sequence of distinct events occurs in succession and leads to the perpetuation of the disease and the pathogen. Therefore, the disease cycle involves survival and dissemination of the phytopathogen and infection of the host (Agrios, 1988; Goto, 1992).

A disease cycle may partially coincide with the life cycle of the pathogen, but it principally defines the appearance, development, and perpetuation of the disease in relation to the pathogen. Thus, the disease cycle does not refer to the pathogen itself. Rather, it involves disease symptoms and changes in both the host plant and pathogen. It spans periods within the growing season and from one growing season to the next. The primary events occurring within a disease cycle are host inoculation, penetration, and infection; and pathogen invasion, colonization, and dissemination (Agrios, 1988).

Inoculation refers to a bacterial phytopathogen coming into contact with a host plant. Pathogens which have become established within a plant and then give rise to disease are called the inoculum. However, an infectious disease will only result if the phytopathogenic bacteria can penetrate the host plant. Bacteria never enter the host directly since they are incapable of passing directly across the epidermis of the host plant. Therefore, natural openings and wounds are considered to be the main avenues for the penetration of bacteria into plants (Dickinson and Lucas, 1982; Agrios, 1988; Goto, 1992).

After the initial entry of a pathogen into a host, the infection process begins. In this phase of the disease cycle, the bacteria establish contact with the susceptible cells or tissues and begin to obtain nutrients from them. Invasion of plant tissues by phytopathogenic bacteria occurs intercellularly and refers to the development of disease following infection. This invasion process is then itself followed by the colonization process in which the bacteria reproduce and establish themselves throughout the host tissues (Dickinson and Lucas, 1982; Agrios, 1988; Goto, 1992). This progress of the bacteria throughout the plant is achieved by the maceration of tissues (Fox et al., 1971; Fox et al., 1972; Dickinson and Lucas, 1982) or the use of natural channels such as phloem and xylem vessels (Wallis et al., 1973; Goodman and White, 1981; Suhayda and Goodman, 1981; Dickinson and Lucas, 1982). The disease cycle concludes when the phytopathogenic bacteria are passively dispersed from diseased plant tissues by various agents such as water, wind, insects, animals, and humans (Agrios, 1988; Goto, 1992).

2.11 KOCH'S POSTULATES

One of the initial procedures in determining whether a specific plant disease is caused by a particular phytopathogenic bacteria would be to attempt the isolation of bacterial cells from diseased tissue and to culture these cells in the laboratory. However, phytopathogenic bacteria are not restricted in their habitats to only host plants. They also occur extensively throughout the environment as epiphytes and saprophytes (Agrios, 1988; Goto, 1992; Dickinson and Lucas, 1982). Hence, the association of a particular bacterium with a diseased plant does not indicate that the isolated bacteria is, in fact, the causal agent of the disease.

To determine whether a specific bacterial isolate is the causal agent of the disease rather than a contaminant, it is imperative that experimental evidence is obtained on the bacterium-host relationship. In 1876, Robert Koch experimentally outlined and defined the criteria for the designation of a disease agent. He established a set of experimental conditions which must be fulfilled in order to establish that a given organism is the causal agent of a specific disease. Koch's criteria of pathogenicity have become known as Koch's postulates and can be summarized as follows (Agrios, 1988; Dickinson and Lucas, 1982; Nester et al., 1995):

1. Without exception, the organism must always be associated with the diseased tissue or plant being examined.
2. The organism must be isolated and grown in pure culture, without any other organisms being present, and its characteristics must be described.
3. The cultured organism must be inoculated into healthy, susceptible plants of the same species or variety from which it was originally isolated, and it must produce the same disease on the inoculated plants as originally observed on the initial host plants.
4. The organism must be reisolated in pure culture from the experimental test plants and its characteristics must be identical to those observed for the organism initially isolated (postulate 2).

If all of Koch's postulates have been experimentally satisfied, then the particular bacterial isolate is the causal agent of the disease (Agrios, 1988).

2.12 PSEUDOMONAD PATHOGENS OF THE *Brassica* SPECIES

2.12.1 *Pseudomonad pathogens of the crop brassicas*

P. syringae pv. *syringae* is a notorious phytopathogen of numerous, unrelated hosts (Palleroni, 1984; Bradbury, 1986; Agrios, 1988). In particular, it has been reported that *Brassica rapa* variety (var.) *pekinensis* (bok choy) is a host for *P. syringae* pv. *syringae* (Bradbury, 1986). The crucifer pathogen, *Pseudomonas syringae* pv. *maculicola*, is a common phytopathogen of the crop brassicas broccoli (*Brassica oleracea* var. *italica*), cabbage (*Brassica oleracea* var. *capitata*), and cauliflower (*Brassica oleracea* var. *botrytis*) (Palleroni, 1984).

P. syringae pv. *maculicola* has also been isolated from all the following crucifers: *Brassica juncea* (oriental mustard), *Brassica nigra* (black mustard), *Brassica oleracea* var. *acephala* (kale), *B. rapa* var. *pekinensis* (bok choy), and *Raphanus sativus* (radish) (Henderson et al., 1992; Wiebe and Campbell, 1993; Cuppels and Ainsworth, 1995). *B. juncea*, *B. oleracea* (varieties *acephala*, *botrytis*, *capitata*, and *italica*), and *R. sativus* have also been shown to be infected by several strains of *P. syringae* pv. *maculicola* under laboratory conditions (Henderson et al., 1992; Wiebe and Campbell, 1993).

P. syringae pv. *tomato* and *P. syringae* pv. *maculicola* are closely related species (Hildebrand et al., 1982; Henderson et al., 1992; Cuppels and Ainsworth, 1995). Accordingly, it has also been established that several strains of *P. syringae* pv. *tomato* are virulent on cabbage, cauliflower, kale, and oriental mustard under laboratory conditions (Henderson et al., 1992; Cuppels and Ainsworth, 1995).

2.12.2 *Pseudomonad pathogens of canola*

Although *P. syringae* pv. *syringae* and *P. syringae* pv. *maculicola* are common phytopathogens of various crop brassicas, there have been no reports of these bacteria naturally infecting canola (*Brassica napus* and *Brassica rapa*). Consequently, the very prevalent and destructive pseudomonads do not seriously affect canola. These bacteria

are not considered agronomically important pathogens of *B. napus* or *B. rapa*.

Nevertheless, there are a few reports of various *Pseudomonas* species infecting canola under laboratory and field conditions.

Sixteen rhizosphere *Pseudomonas* field isolates have been shown to have strong pathogenic activity on *B. napus* Linnaeus (L.) cultivar (cv.) Niklas when they were applied to the plant roots. This treatment rapidly caused mottling, chlorosis, and leaf distortion. Nevertheless, the initially affected plants tended to recover and assume normal growth and appearance (Gerhardson et al., 1985). *P. fluorescens* isolate Å112 and a taxonomically, uncharacterized *Pseudomonas* isolate (*Pseudomonas* Å313) have also been determined to be deleterious rhizobacteria on several *B. napus* and *B. rapa* cultivars (Åström, 1991).

It has also been established that a field-isolated, fluorescent, rhizosphere pseudomonad (*Pseudomonas* rp2) is pathogenic on *B. rapa* cultivars. If *Pseudomonas* rp2 is present on *B. rapa* L. cv. Candle or cv. Tobin seeds at the time of sprouting, it causes inhibition of root growth leading to death or delayed maturation of the plant (Campbell et al., 1986). Furthermore, *P. solanacearum* has been shown to be the causal agent of an uncharacterized wilt disease of *B. rapa* L. cv. Toria in India (Singh, 1992). Finally, several strains of *P. syringae* pv. *maculicola* infect *B. napus* and *B. rapa* under laboratory conditions (Henderson et al., 1992; Wiebe and Campbell, 1993).

2.12.3 Bacterial pod spot of canola

The only suitably characterized, naturally occurring *Pseudomonas* disease of canola is bacterial pod spot. An undescribed bacterial disease of canola (*B. rapa*) was found on two farms in southern Alberta in 1973 (Henry, 1974). The causal bacterium was shown to be a species of *Pseudomonas* and damage to the crops was reported to be as high as 50%. Specifically, the canola pods were directly attacked by the bacterium and many of these diseased pods failed to develop properly and produce normal seed. This initial study indicated that the bacterium was primarily a wound parasite and infection occurred almost exclusively in wounded areas (Henry, 1974).

This work was followed by a more extensive study in 1977 (Henry and Letal, 1977). In this report, it was stated that bacterial pod spot of canola had since been found throughout Alberta and appeared to be quite widespread. The causal bacterium was identified as a strain of *P. syringae* (Henry and Letal, 1977).

Four varieties of both *B. rapa* and *B. napus* were proven to be susceptible to the pathogen. In addition, it was shown that the *B. rapa* and *B. napus* varieties were all equally susceptible and no marked resistance could be detected in any variety. The researchers stressed that the wounding of the host was an important predisposing factor in the infection by *P. syringae*. It was suggested that the wounding of canola by frost and insects plays an important role in the development of bacterial pod spot and also partially accounts for the occurrence and severity of the disease (Henry and Letal, 1977).

2.13 THE RELATIONSHIP BETWEEN *Pseudomonas syringae* pv. *maculicola* AND *Pseudomonas syringae* pv. *tomato*

P. syringae pv. *maculicola* and *P. syringae* pv. *tomato* have always been considered separate but closely related species. Nevertheless, it was recently proposed that these two pathovars be combined into one species based on physiological and pathological analyses (Takikawa et al., 1994). Restriction fragment length polymorphism (RFLP) analysis of total DNA has provided evidence that supports a taxonomic fusion of these two pathovars by revealing that they are two of the most closely related pathovars of *P. syringae* (Denny, 1988b). Additionally, bacteria identified as *P. syringae* pv. *maculicola* and those identified as *P. syringae* pv. *tomato* have very similar nutritional profiles and phenotypic characteristics (Hildebrand et al., 1982; Henderson et al., 1992; Cuppels and Ainsworth, 1995). *P. syringae* pathovars *maculicola* and *tomato* also appear to have overlapping host ranges and they share the same RFLP patterns when hybridized with various probes (Henderson et al., 1992; Cuppels and Ainsworth, 1995). Consequently, *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato* may represent a single genetic group (Palleroni, 1984).

Henderson et al. (1992) analyzed the RFLP patterns, nutritional spectra, and

host ranges of *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato* and found no distinctive profiles that could conclusively differentiate the strains included in their study. Their analyses indicated that these pathovars constitute a single genetic group with several subgroups. In addition, the subgroups are not unique as each subgroup possesses characteristics of one or more of the other subgroups (Henderson et al., 1992). The pathogenicity, coronatine production, carbohydrate utilization, plasmid profile, and RFLP pattern data presented by Cuppels and Ainsworth (1995) also support this conclusion.

Nevertheless, Henderson et al. (1992) were able to categorize the *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato* strains analyzed in their study into four subgroups based on phenotypic characterizations and RFLP analysis with a rutin glycosidase gene probe. These four subgroups also appear to represent diverging genetic groups that still share genetic information relating to pathogenicity and host range (Henderson et al., 1992). Therefore, until the *P. syringae* pv. *maculicola/tomato* genetic group has been taxonomically clarified, these two pathovars should remain separate.

2.14 THE CROP BRASSICAS

2.14.1 Introduction

The crop brassicas, which include canola (*B. napus* and *B. rapa*), belong to the division *Tracheophyta*, class *Angiospermae*, subclass *Dicotyledonae*, order *Rhoeadales*, family *Cruciferae* of kingdom *Plantae* (Gill and Vear, 1980). The *Cruciferae* family contains several important crop plants besides the oilseed brassicas *B. napus* and *B. rapa*, such as bok choy, broccoli, brussels sprouts, cabbage, kale, mustard, radish, rutabaga, and turnip. Because the word rape is derived from the Latin word rapum, meaning turnip, the oilseed Brassicas are commonly referred to as rapeseed crops (Downey et al., 1974; Downey et al., 1975; Kneen, 1992; Röbbelen et al., 1989).

Brassica and other closely related cruciferous crops are widely cultivated as vegetable crops in nations throughout the world. These crops are grown for human

consumption, as condiments and spices, and as fodder crops for livestock feeding. However, these crops are cultivated to the greatest extent for edible vegetable oil production (Downey and Rimmer, 1993). Over 13.2% of the world's edible oil supply is now derived from the oilseed brassicas, rapeseed and mustard. In fact, the production and utilization of *Brassica* seed oils has grown faster in the years 1975 to 1985 than any other crop, except the oil palm, making it the third most important edible oil source after soybean and palm (Downey and Röbbelen, 1989).

The oilseed brassicas are well adapted to germinate and grow in cool, moist environments. Due to this ability to produce high yields at low temperatures, the brassicas can be cultivated as spring-sown crops in the cool and high-elevation agricultural regions. Accordingly, these crops can also be cultivated as winter crops in the more temperate zones. Consequently, the major oilseed *Brassica*-producing regions include Canada, China, northern Europe, and the Indian subcontinent (Downey and Rakow, 1991; Downey and Röbbelen, 1989).

2.14.2 Canola in Canada

Since the 1940s, an extraordinary increase in *Brassica* oilseed production has occurred worldwide. In Canada, this increase in production was associated with seed quality improvements through plant breeding programs that modified the fatty acid composition (elimination of erucic acid) and the reduction of glucosinolate content in the residual meal. As a result, in the years from 1948 to 1952, 70% of the total world oilseed *Brassica* production of 2.8 million tones was produced in Asia, but by 1984, Canada (20%) and Europe (35%) produced more than half the total world production of 15.9 million tones. Oilseed brassicas account for roughly 10% of the total world oilseed production and 15% of the total edible vegetable oil production (Downey and Rimmer, 1993).

In North America the term canola has been used to describe cultivars that have less than 2% erucic acid as a percentage of total fatty acids in the extracted seed oil and an aliphatic glucosinolate content in the residual meal of less than $30 \mu\text{mol}\cdot\text{g}^{-1}$. Today there are only two species (*B. napus* and *B. rapa*) that have been bred with

both low-erucic and low-glucosinolate (double-low) quality, and these species are now extensively grown commercially within Canada (Downey and Rimmer, 1993; Downey and Rakow, 1991). It is now generally recognized that the *B. napus* L. species includes both Argentine type rape and rutabagas, while the *B. rapa* L. (synonym *B. campestris*) species contains both Polish type rape and turnips (Downey et al., 1974).

2.14.3 Genomic relationships of the crop brassicas

Interspecific hybridization is a frequent process among the angiosperms and ferns. The origin of a new species directly from a new hybrid commonly demands some mechanism for stabilizing the breeding behavior of the hybrid. Perhaps the most typical mode of hybrid speciation is amphiploidy which accounts for the majority of polyploid species and 30 - 50% of all angiosperm species (Grant, 1981). In this process fertility is restored to a sterile hybrid by the doubling of both chromosome sets either through the sexual functioning of cytologically non-reduced gametes or through the spontaneous doubling of the somatic chromosomes (Fehr, 1991).

Original cytogenic investigations revealed that crop brassicas are composed of the three elementary diploid species; *B. rapa* ($2n = 20$; AA), *B. nigra* ($2n = 16$; BB), and *B. oleracea* ($2n = 18$; CC), and three amphidiploids which arose via interspecific hybridization between any two of the three elementary diploid species. *B. napus* ($2n = 38$; AACC), *B. juncea* ($2n = 36$; AABB), and *Brassica carinata* ($2n = 34$; BBCC) constitute the amphidiploid group of the brassicas (Labana and Gupta, 1993). The cytogenic relationships between the *Brassica* species was demonstrated by U (1935) and have become known as the U-triangle (Figure 2.3). This evolutionary relationship between the brassicas has since been confirmed by several other studies and taxonomic criteria such as flavonoid composition (Dass and Nybom, 1967), nuclear DNA content (Verma and Rees, 1974), seed protein serology (Vaughan, 1977), chromosome pairing and artificial synthesis of amphidiploids (Prakash and Hinata, 1980), DNA analysis (Erickson et al., 1983), mutational analysis (Palmer et al., 1983), isozyme analysis (Quiros et al., 1985), RFLP analysis (Song et al., 1988), and the use of genome-specific chromosome markers (Hosaka et al., 1990). The close homology between the

elementary diploid species has been identified by homologous pairing (Attia and Röbbelen, 1986) and by the presence of shared fragments between the A, B, and C genomes (Hosaka et al., 1990).

2.15 OBJECTIVES OF THESIS

This thesis questioned whether it is possible to isolate unidentified *R* genes from agronomically important crops using PCR with degenerate oligonucleotide primers based on the protein sequence of an *R* gene product from a distantly related crop species. The goals of this thesis were (i) to determine the presence of *Pto* homologs in *B. napus* by isolating them with a PCR-based approach, (ii) to ascertain the presence of an ORF containing serine/threonine protein kinase catalytic domain sequence motifs internal to those used as priming sites, (iii) to prove that *P. syringae* pv. *tomato* is a causal agent of disease in *B. napus*, (iv) to demonstrate cosegregation of the *B. napus* *Pto* homolog with a phenotypically defined *P. syringae* pv. *tomato* resistance gene or fenthion sensitivity gene, and (v) to identify any intragenomic differences within the crop brassicas with regards to *R* genes by analyzing RFLP patterns on Southern blots. These objectives were investigated since the isolation of *R* genes based solely on amino acid sequence data will provide fundamental knowledge of the function of *R* genes in disease resistance signalling pathways. Furthermore, this PCR-based approach to cloning *R* genes will promote advances in plant breeding by providing probes for marker assisted selection and transgenic strategies for disease control.

Table 2.1. The Five Classes of Cloned Plant Disease Resistance Genes^a.

Class	<i>R</i> gene	Plant	Pathogen	<i>Avr</i> gene	Structure ^b	Ref. ^c
1	<i>Pto</i>	Tomato	<i>Pseudomonas syringae</i> pv. <i>tomato</i>	<i>avrPto</i>	PK	(1)
2a	<i>RPS2</i>	<i>Arabidopsis</i>	<i>P. syringae</i> pv. <i>tomato</i>	<i>avrRpt2</i>	LZ-NBS-LRR	(2, 3)
	<i>RPM1</i>	<i>Arabidopsis</i>	<i>P. syringae</i> pv. <i>maculicola</i>	<i>avrRpm1</i> and <i>avrB</i>	LZ-NBS-LRR	(4)
	<i>Prf</i>	Tomato	<i>P. syringae</i> pv. <i>tomato</i>	<i>avrPto</i>	LZ-NBS-LRR	(5)
	<i>I2C-1</i>	Tomato	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> race 2	Unknown	NBS-LRR-LZ	(6)
2b	<i>N</i>	Tobacco	Tobacco mosaic virus	Unknown	TIR-NBS-LRR	(7)
	<i>L6</i>	Flax	<i>Melampsora lini</i>	Unknown	TIR-NBS-LRR	(8)
	<i>M</i>	Flax	<i>M. lini</i>	Unknown	TIR-NBS-LRR	(9)
	<i>RPP5</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	Unknown	TIR-NBS-LRR	(10)
3	<i>Cf-9</i>	Tomato	<i>Cladosporium fulvum</i>	<i>Avr9</i>	LRR-TM	(11)
	<i>Cf-2</i>	Tomato	<i>C. fulvum</i>	<i>Avr2</i>	LRR-TM	(12)
	<i>Hs1^{pro-1}</i>	Sugar beet	<i>Heterodera schachtii</i>	Unknown	LRR-TM	(13)
4	<i>Xa21</i>	Rice	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	Unknown	LRR-TM-PK	(14)
5	<i>HM1</i>	Maize	<i>Cochliobolus carbonum</i> race 1	None	Toxin reductase	(15)

^a Adapted from Baker et al. (1997); and Hammond-Kosack and Jones (1997).

^b Structure refers to the structural motifs predicted by analysis of the deduced amino acid sequences of the listed genes: protein kinase (PK); leucine zipper (LZ); nucleotide binding site (NBS); leucine-rich repeat (LRR); sequence homologous to the cytoplasmic domains of Toll and Il-1R (TIR); and transmembrane domain (TM). See text for further details.

^c Reference(s) reporting the cloning and characterization of the *R* gene. (1) Martin et al. (1993a); (2, 3) Bent et al. (1994), Mindrinos et al. (1994); (4) Grant et al. (1995); (5) Salmeron et al. (1996); (6) Ori et al. (1997); (7) Whitham et al. (1994); (8) Lawrence et al. (1995); (9) Anderson et al. (1997); (10) Parker et al. (1997); (11) Jones et al. (1994); (12) Dixon et al. (1996); (13) Cai et al. (1997); (14) Song et al. (1995); and (15) Johal and Briggs (1992).

		Host plant genotype			
		R_1, R_2	R_1, r_2	r_1, R_2	r_1, r_2
Pathogen genotype	<i>Avr1, Avr2</i>	—	—	—	+
	<i>Avr1, avr2</i>	—	—	+	+
	<i>avr1, Avr2</i>	—	+	—	+
	<i>avr1, avr2</i>	+	+	+	+

Figure 2.1. Gene-for-Gene Interactions Between Plants and Pathogens.

R_1 and R_2 represent two dominant plant resistance genes while r_1 and r_2 represent their recessive, nonfunctional alleles. The R_1 and R_2 gene products permit plants to recognize pathogens carrying the corresponding, dominant *Avr1* and *Avr2* avirulence genes but not the recessive *avr1* and *avr2* alleles. A compatible interaction that results in disease only occurs if the plant lacks a resistance gene or possesses the nonfunctional allele (r_1 or r_2); or the pathogen lacks the corresponding avirulence gene or possesses the recessive allele (*avr1* or *avr2*). The (—) sign denotes an incompatible interaction where the plant is resistant to the pathogen while the (+) sign denotes a compatible interaction where the plant is susceptible to the pathogen. Adapted from Agrios (1988); Briggs and Johal (1994); Jones (1994); and Hammond-Kosack and Jones (1997).

		Host plant genotype	
		Rx	rr
Pathogen	<i>Com</i>	—	+
genotype	<i>com</i>	—	—

Figure 2.2. Toxin-dependent Interactions Between Plants and Pathogens.

The wild-type *Com* gene of the pathogen is required for the synthesis of a toxin compatibility factor that is essential for pathogenesis. The corresponding recessive, nonfunctional allele is represented by *com*. The dominant plant R gene is required for resistance since it detoxifies the pathogen-produced compatibility factor. However, resistance can also be achieved if the plant expresses a toxin-insensitive form of the toxin target molecule. Colonization only occurs during compatible interactions when the pathogen produces a functional toxin that the plant is incapable of detoxifying. The (—) sign denotes an incompatible interaction where the plant is resistant to the pathogen while the (+) sign denotes a compatible interaction where the plant is susceptible to the pathogen. Adapted from Agrios (1988); Briggs and Johal (1994); Jones (1994); and Hammond-Kosack and Jones (1997).

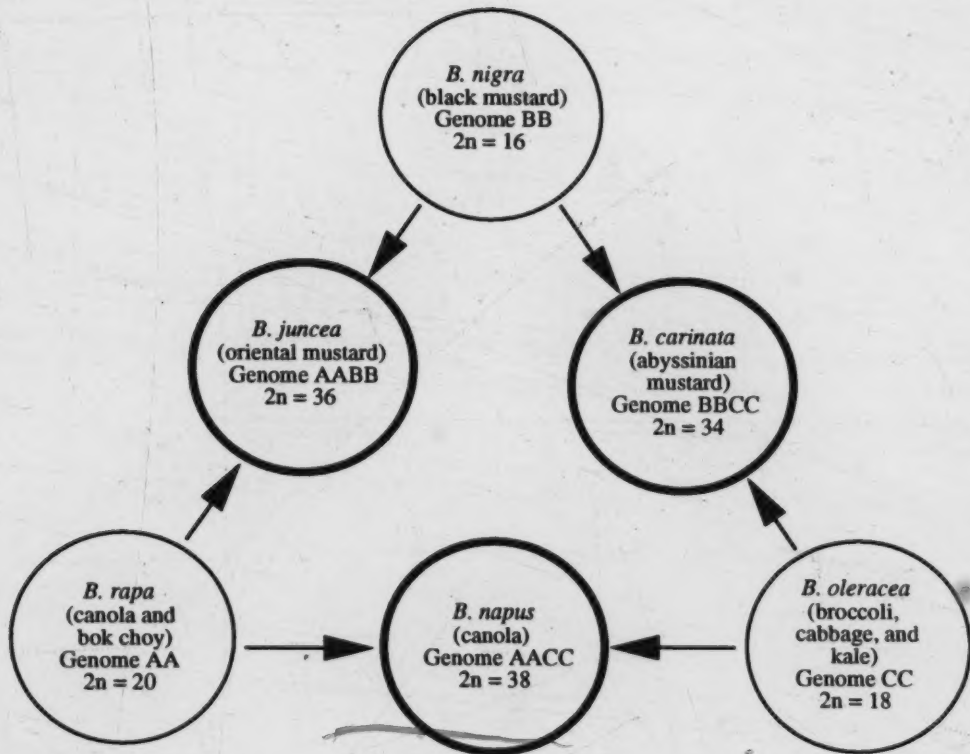


Figure 2.3. Genomic Relationships of the Crop Brassicas.

The crop brassicas are composed of the three elementary diploid species, *Brassica rapa*, *B. nigra*, and *B. oleracea*; and the three amphidiploid species, *B. napus*, *B. juncea*, and *B. carinata*. The amphidiploids arose via interspecific hybridization between any two of the elementary diploid species as illustrated in the figure. Adapted from U (1935).

3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

The plant species, accessions, breeding lines, and cultivars used in this study are listed in Table 3.1. Plants were cultivated 2 per pot in sterile Metro-Mix 290 (W.R. Grace & Co. of Canada Ltd., Ajax, ON, Canada) in environmental growth chambers under a 16 hour photoperiod with a temperature regime of 21°C/18°C (day/night), a light intensity of 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a relative humidity of 50%-60%. Illumination was provided by a combination of 40 W incandescent bulbs and 215 W very high output (VHO) cool white fluorescent tubes. Plants were watered as required and, beginning 10 days after planting, they were fertilized weekly with instant soluble 20:20:20 (N:P:K) fertilizer applied at a concentration of 200 mgL^{-1} .

3.2 SURFACE STERILIZATION OF SEEDS

Prior to planting, all seeds were soaked with continuous shaking in 70% (v/v) ethanol for 5 minutes. The seeds were then soaked with continuous shaking in 0.525% (v/v) sodium hypochlorite [10% (v/v) Javex bleach] and 0.5% sodium dodecylsulfate (SDS) for 15 minutes. Following these sterilization steps, the seeds were washed three times in sterile double distilled water (ddH_2O) with continuous shaking for 10 minutes each wash. The seeds were then cultivated as described above.

3.3 BACTERIAL STRAINS, MEDIA, AND GROWTH CONDITIONS

The *P. syringae* pathovars, isolates, and strains used in this study are listed in Table 3.2. The *P. syringae* isolates were routinely grown at 28°C on nutrient broth-yeast extract (NBY) agar (Vidaver, 1967), in NBY broth, on King's medium B agar (King et al., 1954), on KBC medium agar (Mohan and Schaad, 1987), in KBC medium broth, on Vogel-Bonner-tartrate (VB-tar) agar (Davis et al., 1980; Cuppels, 1986; Cuppels, 1988; Cuppels et al., 1990), and in VB-tar broth. VB-tar consisted of modified Vogel-Bonner minimal medium (Davis et al., 1980; Cuppels, 1986) in which

0.3% (w/v) D(-)-tartaric acid (Sigma Chemical Co.) replaced the 0.2% (w/v) citric acid (Cuppels et al., 1990). In addition, VB-tar also contained 9.3×10^{-6} M FeCl_3 [2.5×10^{-4} % (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; Cuppels, 1988; Dr. D.A. Cuppels, personal communication].

When required, the media used for *P. syringae* growth were supplemented with one or more of the following filter-sterilized antibiotics: cephalixin (80 $\mu\text{g/mL}$), cycloheximide (200 $\mu\text{g/mL}$), kanamycin (25 $\mu\text{g/mL}$), rifampicin (100 $\mu\text{g/mL}$), spectinomycin (20 $\mu\text{g/mL}$), and tetracycline (10 $\mu\text{g/mL}$). *E. coli* strains JM109 and DH5 α F' were utilized in the bacterial transformation experiments and they were grown at 37°C on Luria-Bertani-Miller (LB-Miller) medium (Sambrook et al., 1989) or in LB-Miller broth containing 100 $\mu\text{g/mL}$ ampicillin.

3.4 PRESERVATION OF CULTURES

All bacterial isolates and strains were placed in low-temperature storage to maintain viability and pathogenicity. Prior to low-temperature storage, pure *P. syringae* cultures were selectively grown at 28°C in NBY broth supplemented with 100 $\mu\text{g/mL}$ rifampicin (*P. syringae* pv. *tomato* strains DC3000 and T1); in NBY broth supplemented with 25 $\mu\text{g/mL}$ kanamycin and 100 $\mu\text{g/mL}$ rifampicin [*P. syringae* pv. *tomato* strain T1(pPtE6)]; in NBY broth supplemented with 100 $\mu\text{g/mL}$ rifampicin and 10 $\mu\text{g/mL}$ tetracycline [*P. syringae* pv. *tomato* strain DC3000(pLH12)]; in NBY broth supplemented with 100 $\mu\text{g/mL}$ rifampicin, 20 $\mu\text{g/mL}$ spectinomycin, and 10 $\mu\text{g/mL}$ tetracycline [*P. syringae* pv. *tomato* strain DC3000(pLH12 Ω)]; in KBC broth (*P. syringae* pv. *syringae* isolates Crak and Crok); and in VB-tar broth (*P. syringae* pv. *maculicola* strain 84-59). Additionally, pure *E. coli* (strains JM109 and DH5 α F') cultures were selectively grown at 37°C in LB-Miller broth supplemented with 100 $\mu\text{g/mL}$ ampicillin. All cultures were grown to saturation and, immediately after growth, the fresh cultures were stored at -80°C in 7% (v/v) dimethylsulfoxide (DMSO) according to Lech and Brent (1989a) and Davis et al. (1980).

3.5 PLANT PATHOGENICITY TESTS

3.5.1 Preparation of the inoculum

Bacterial isolates and strains were grown on selective media and a single colony was then used to inoculate NBY broth (Vidaver, 1967). These liquid NBY cultures were grown for 16 hours at 28°C. The bacterial cells were pelleted by centrifugation (13,000xg for 2 min at 4°C) and washed three times with sterile ddH₂O. The pelleted cells were then resuspended in 1 mL of sterile ddH₂O. An optical density reading at 600 nm was determined for a dilution of the resuspended pellet and the concentration of bacterial cells was estimated according to Lech and Brent (1989b). The resuspended pellet solution was then used to prepare an inoculum of either 1×10^8 or 5×10^6 colony forming units (cfu) per mL in sterile ddH₂O.

3.5.2 Preliminary phytopathogen screening

Four-week-old plants of the *B. napus* L. breeding lines, *B. napus* L. cultivars, and *B. rapa* L. variety (var.) *oleifera* cultivars listed in Table 3.3 were used in preliminary bacterial phytopathogen screening experiments. Using sterile technique, eight plants from each of these breeding lines and cultivars (Table 3.3) were inoculated with each of the *P. syringae* pathovars, strains, and isolates listed in Table 3.2. These plants were inoculated with the phytopathogens by gently rubbing the first 3 true leaves with a cotton swab covered in carborundum, rinsing off the carborundum with ddH₂O, and then rubbing the injured leaves with a cotton swab moistened with a bacterial suspension (1×10^8 cfu/mL). This high inoculum level was employed for these preliminary phytopathogen studies since there had never been any published reports of pathogenicity studies using these bacteria and *B. napus* or *B. rapa*. Consequently, this high dosage was expected to lead to an infection in susceptible host plants. Two replicates of this experiment were performed.

Eight 4-week-old plants of each *B. napus* L. breeding line, *B. napus* L. cultivar and *B. rapa* L. var. *oleifera* cultivar (Table 3.3) were also used as negative treatment controls in each experiment by following the above procedure except they were

exposed to sterile ddH₂O and an autoclaved 1×10^8 cfu/mL bacterial suspension instead of the viable inoculum. Moreover, in each experiment, 4-week-old *L. esculentum* cv. Bonny Best, cv. Moneymaker, and/or cv. Rio Grande-PtoR plants acted as positive and negative controls for the pathogenicity of the bacteria. Four tomato plants from each cultivar were inoculated by gently rubbing the first 5 true leaves with a sterile cotton swab moistened with a bacterial suspension (1×10^8 cfu/mL). Four plants of each tomato cultivar were also used as negative treatment controls in each experiment by following the above procedure except they were exposed to sterile ddH₂O and an autoclaved 1×10^8 cfu/mL bacterial suspension instead of the viable inoculum.

All of the inoculated and control canola and tomato plants were then misted with sterile ddH₂O and immediately covered with clear plastic bags that were placed over wire frames to prevent the plants from touching the bags. The inoculated plants were then grown under the environmental conditions described in section 3.1. The plants were incubated in the plastic bags for 3 days after which time the bags were removed. The development of an HR or disease symptoms was monitored visually for a period of 10 days after inoculation and control treatment.

Four-week-old plants of the *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars listed in Table 3.4 were used in additional bacterial phytopathogen screening experiments. Eight plants from each of these cultivars (Table 3.4) were inoculated with each of the following bacterial strains: (1) *P. syringae* pv. *tomato* strain DC3000(pLH12), (2) *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω), (3) *P. syringae* pv. *tomato* strain T1, and (4) *P. syringae* pv. *tomato* strain T1(pPtE6). The plants were inoculated by following the aforementioned inoculation procedure except a bacterial inoculum of 5×10^6 cfu/mL was used.

This decreased inoculum level was used to prevent false positive reactions due to high dosages of inoculum. That is, an inoculum level of 1×10^8 cfu/mL may be too high and, as a result, may cause an assortment of reactions on non-host plants. These variable reactions can then lead to a misinterpretation of host ranges (Hildebrand et al., 1988).

Four 4-week-old plants of each *B. napus* and *B. rapa* L. var. *oleifera* cultivar

(Table 3.4) were also used as negative treatment controls in each experiment by following the aforementioned control procedure except they were exposed to sterile ddH₂O and an autoclaved 5×10^6 cfu/mL bacterial suspension instead. Furthermore, in each experiment 4-week-old *L. esculentum* cv. Bonny Best, cv. Moneymaker, and cv. Rio Grande-PtoR plants acted as positive and negative controls for the pathogenicity of the *P. syringae* pv. *tomato* strains. These tomato plants were inoculated with a 5×10^6 cfu/mL bacterial suspension, an autoclaved 5×10^6 cfu/mL bacterial suspension, and sterile ddH₂O as described above in the tomato control treatment procedure.

All of the inoculated and control plants were grown as previously described. The development of an HR or disease symptoms was monitored visually for a period of 14 days after inoculation and control treatment.

3.5.3 Determinative pathogenicity study

For a determinative pathogenicity study, fourteen 3-week-old *B. napus* L. cv. Polo and cv. Westar plants were inoculated with *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) as described in section 3.5.2 except a bacterial inoculum of 5×10^6 cfu/mL was used instead. Four 3-week-old *B. napus* L. cv. Polo and cv. Westar plants were also used as negative treatment controls by following the inoculation procedure except they were exposed to sterile ddH₂O instead of the viable inoculum. Four 3-week-old *L. esculentum* cv. Bonny Best plants acted as positive controls for the pathogenicity of *Pst* DC3000 and were inoculated with a cell suspension of 5×10^6 cfu/mL as described in section 3.5.2. Additionally, four 3-week-old *L. esculentum* cv. Bonny Best plants were used as negative treatment controls by following the above procedure except they were exposed to sterile ddH₂O instead of the viable inoculum.

All of the inoculated and control *B. napus* L. and *L. esculentum* plants were then misted with sterile ddH₂O and immediately placed in clear plastic humidity chambers. Separate humidity chambers were used for the inoculated and control plants. Both the inoculated and control plants were then grown under a 16 hour photoperiod with a temperature regime of 21°C/18°C (day/night), a light intensity of $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, and a relative humidity of 90% to simulate cloudy and rainy weather.

After three days in the humidity chambers, the inoculated and control plants were removed and grown under the environmental conditions described in section 3.1. The development of an HR or disease symptoms was monitored visually for a period of 12 days after inoculation and control treatment. Three replicates of this experiment were performed.

3.5.4 Verification of Koch's postulates

To prove that *Pst* DC3000 was a causal agent of disease in *B. napus* L. cv. Westar, Koch's postulates had to be experimentally satisfied (Agrios, 1988). Therefore, twenty 3-week-old *B. napus* cv. Westar plants were inoculated with a bacterial suspension. The inoculum consisted of a pure bacterial culture isolated from infected *B. napus* L. cv. Westar leaf tissue that was inoculated with *Pst* DC3000 in replicate experiment two of the determinative pathogenicity study (see section 3.5.3 above and section 3.7 below). The plants were inoculated with the method described in section 3.5.2 except a bacterial inoculum of 5×10^6 cfu/mL was used instead.

Sixteen 3-week-old *B. napus* L. cv. Westar plants were also used as negative treatment controls by following the inoculation procedure except they were exposed to sterile ddH₂O instead of the viable inoculum. Four 3-week-old *L. esculentum* cv. Bonny Best plants acted as positive controls for the pathogenicity of the bacterial isolate described above and were inoculated with a cell suspension of 5×10^6 cfu/mL as outlined in section 3.5.2. In addition, four 3-week-old *L. esculentum* cv. Bonny Best plants were used as negative treatment controls by following the above procedure except they were exposed to sterile ddH₂O instead of the viable inoculum. All of the inoculated and control *B. napus* L. cv. Westar and *L. esculentum* cv. Bonny Best plants were then treated and grown exactly as described in section 3.5.3 above (determinative pathogenicity study). The development of an HR or disease symptoms was monitored visually for a period of 12 days after inoculation and control treatment.

3.6 DETERMINATION OF *in planta* BACTERIAL GROWTH

For the determinative pathogenicity study (section 3.5.3), the levels of *Pst*

DC3000 in the leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. cv. Westar were determined prior to inoculation; immediately after inoculation (day 0); and 1, 2, 4, 6, and 8 days after inoculation. Bacterial populations were sampled by taking three leaf discs from each plant (1 from each inoculated leaf) using a number 4 cork borer (0.85 cm diameter) and macerating these leaf discs in 400 μ L of sterile 10 mM MgCl_2 . The density of bacterial populations (cfu/cm²) was then determined by plating the appropriate serial dilutions on fresh King's medium B agar (King et al., 1954) containing 100 μ g/mL rifampicin and counting the number of colony-forming units. Two plants of each cultivar were sampled at each time point and three replicate experiments were performed. All plant material was thoroughly washed with water prior to sampling.

During the pathogenicity study used to verify Koch's postulates (section 5.3.4), the levels of the bacterial isolate in the leaves of *B. napus* L. cv. Westar were determined 8 days after inoculation. Bacterial populations were sampled as described above. Three plants of *B. napus* L. cv. Westar were sampled at 8 days after inoculation. All plant material was thoroughly washed with water prior to sampling.

3.7 CHARACTERIZATION OF BACTERIA ISOLATED FROM INFECTED *Brassica napus* L. cv. WESTAR LEAF TISSUE

3.7.1 Isolation of the bacteria

Three *B. napus* L. cv. Westar plants from 3 different *Pst* DC3000 pathogenicity experiments were used to obtain nine representative bacterial isolates. Specifically, pure bacterial cultures were obtained from 3 infected *B. napus* L. cv. Westar plants from replicate one of the determinative pathogenicity study (section 3.5.3), 3 infected *B. napus* L. cv. Westar plants from replicate two of the determinative pathogenicity study (section 3.5.3), and 3 infected *B. napus* L. cv. Westar plants from the pathogenicity study used to verify Koch's postulates (section 5.3.4). It must be noted that the 6 infected *B. napus* L. cv. Westar plants from replicates one and two of the determinative pathogenicity study were inoculated with *Pst* DC3000 (section 3.5.3).

However, the 3 infected *B. napus* L. cv. Westar plants from the pathogenicity study used to verify Koch's postulates were inoculated with a pure bacterial culture isolated from infected *B. napus* L. cv. Westar leaf tissue that was inoculated with *Pst* DC3000 in replicate experiment two of the determinative pathogenicity study (section 3.5.4). This particular bacterial culture was isolated and purified by the methods described in this section and is, in fact, one of the nine representative bacterial isolates.

All of the nine bacterial isolates were isolated from the *B. napus* L. cv. Westar leaves 8 days after being inoculated and the *Pst* DC3000 isolation method described in section 3.6 was used to obtain these representative isolates. The nine single colonies growing on selective King's medium B agar (100 µg/mL rifampicin) were purified as follows. Single colonies were used to inoculate NBY broth (100 µg/mL rifampicin) and these liquid cultures were streaked onto selective VB-tar agar. Single colonies from these plates were used to inoculate NBY broth (100 µg/mL rifampicin) and these liquid cultures were then streaked onto selective King's medium B agar (100 µg/mL rifampicin). Single colonies from these plates were stored at -80°C in NBY broth (100 µg/mL rifampicin) containing 7% (v/v) DMSO according to Lech and Brent (1989a) and Davis et al. (1980). These purified, long-term stocks were used for all subsequent studies.

Morphological, cultural, physiological, biochemical, hypersensitivity, pathogenicity, coronatine bioassay, and plasmid profile analyses were performed on all of the purified bacterial isolates to differentiate them as fluorescent pseudomonads and, in particular, *P. syringae* pv. *tomato*. All of these numerous tests were also performed on a known *Pst* DC3000 control culture.

3.7.2 Morphological and cultural characters

All of the bacterial isolates and the *Pst* DC3000 control culture were subjected to the Gram stain (Schaad, 1988) to determine their morphology and gram reaction. The bacterial isolates and the *Pst* DC3000 control culture were also subjected to the potassium hydroxide (KOH) test (Suslow et al., 1982) to verify their gram reaction. Following these tests, the purified bacterial strains and the *Pst* DC3000 control culture

were streaked on King's medium B (King et al., 1954), incubated at 28°C for 72 h, and then examined for fluorescein pigment production (Hildebrand et al., 1988). Finally, the ability of these isolates and the *Pst* DC3000 control culture to grow at 41°C was ascertained according to Cuppels (1984).

3.7.3 Physiological and biochemical reactions

The isolates and the *Pst* DC3000 control culture were also tested for production of levan (Hildebrand et al., 1988); and for the presence of catalase (Dickey and Kelman, 1988), oxidase (Kovacs, 1956; Hildebrand et al., 1988; Moore et al., 1988), and arginine dihydrolase (Thornley, 1960; Hildebrand et al., 1988). Hildebrand et al.'s (1988) method was employed to test each bacterial isolate and the *Pst* DC3000 control culture for the ability to ferment nitrogen (ie. the nitrate reduction test). Isolates and the *Pst* DC3000 control culture were also tested for their capacity to form ice nuclei by determining their ice nucleation activity (Hildebrand et al., 1988; Jones et al., 1989; Lindgren et al., 1989).

All of the bacterial isolates and the *Pst* DC3000 control culture were also subjected to the potato soft rot test (Lelliott et al., 1966; Dickey and Kelman, 1988). Furthermore, the ability of the isolates and the *Pst* DC3000 control culture to degrade polypectate at pH 4.6 and 8.5 was determined according to Hildebrand (1971). Finally, the capacity of the isolates and the *Pst* DC3000 control culture to use certain organic substrates as sole carbon sources for growth was determined according to Hildebrand et al. (1988) with the minimal medium of Ayers et al. (1919). The following carbon sources were used in this analysis: i-erythritol (*meso*-erythritol), DL-homoserine (DL-2-amino-4-hydroxybutyric acid), *meso*-inositol, DL-lactic acid, mannitol, D-sorbitol (D-glucitol), and D(-)-tartaric acid.

3.7.4 Hypersensitivity and pathogenicity tests

The hypersensitivity reaction on tobacco leaves was tested with all 9 isolates, the *Pst* DC3000 control culture, and 30-day-old tobacco plants (*Nicotiana tabacum* L. White Gold) (Klement et al., 1964; Cuppels, 1984; Jones et al., 1989). Readings were

made after 24 hours. All of the isolates and the *Pst* DC3000 control culture were tested for pathogenicity on *L. esculentum* cv. Bonny Best by following Cuppels (1986) inoculation method. The inoculated tomato plants were monitored visually for a period of 7 days after inoculation.

3.7.5 Tomato leaf bioassays for coronatine

The tomato leaf chlorosis assay was performed according to Moore et al.'s (1989) procedure which is a modification of the pinprick assay of Woolley et al. (1952). This bioassay was used to monitor the presence of coronatine in culture supernatants of the 9 bacterial isolates and the *Pst* DC3000 control culture. Dried ethyl acetate extracts of the bacterial culture supernatants were dissolved in sterile ddH₂O and a 15 μ L droplet of each solution was applied to the adaxial leaf surfaces of 5-week-old tomato (*L. esculentum* cv. Bonny Best) plants. The ethyl acetate extract solutions were then introduced into the leaflets by gently puncturing the leaf areas covered by the droplets with a sterile stainless-steel pin (Moore et al., 1989). The plants were then incubated for 5 days under environmental conditions described by Cuppels (1986). Following this incubation period, the plants were examined for chlorosis and each chlorotic zone was measured (Moore et al., 1989).

3.7.6 Plasmid DNA isolation and characterization

Prior to plasmid DNA isolation, single colonies of the 9 bacterial isolates and the *Pst* DC3000 control culture were selectively grown at 28°C in NBY (Vidaver, 1967) broth supplemented with 100 μ g/mL of rifampicin. These cultures were then screened for the presence of large indigenous plasmids by using the plasmid detection and isolation protocol of Kado and Liu (1981). However, the alkaline lysate solutions were incubated at 60°C for 60 minutes instead of 20 minutes (Cuppels, 1986). The indigenous plasmids were then separated by horizontal electrophoresis in a 0.45% agarose gel and 1x TBE running buffer (Sambrook et al., 1989; Cuppels and Ainsworth, 1995). The agarose gel was run at approximately 3.3 V/cm for 20 hours (Cuppels and Ainsworth, 1995).

E. coli strain WA803(pGS9) (Selvaraj and Iyer, 1983), *E. coli* strain MM292(pRK2013) (Figurski and Helinski, 1979), *E. coli* strain J5-3(pRP1) (Bukhari et al., 1977), *E. coli* strain J5-3(pR1drd19) (Bukhari et al., 1977), and *E. coli* strain J5-3(pR40a) (Bukhari et al., 1977) were kindly provided by Dr. D.A. Cuppels (Agriculture and Agri-Food Canada, Pest Management Research Centre, London, ON, Canada). The plasmids of these *E. coli* strains were used as reference standards in determining the size of the indigenous plasmids isolated from the 9 bacterial isolates and the *Pst* DC3000 control culture. The sizes of the reference plasmids from *E. coli* strain WA803(pGS9), *E. coli* strain MM292(pRK2013), *E. coli* strain J5-3(pRP1), *E. coli* strain J5-3(pR1drd19), and *E. coli* strain J5-3(pR40a) are 30 kbp, 48 kbp, 57, kbp, 93 kbp, and 146 kbp, respectively.

These *E. coli* strains were selectively grown at 37°C on LB-Miller (Sambrook et al., 1989) broth or in LB-Miller broth supplemented with 50µg/mL of kanamycin. The plasmids of these *E. coli* strains were isolated by Kado and Liu's (1981) method as described directly above. These *E. coli* plasmids were also separated by electrophoresis as described directly above.

3.8 FENTHION AND MALATHION ASSAYS

For preliminary fenthion studies, 5½-week-old plants listed in Table 3.5 were exposed to fenthion (*O,O*-dimethyl *O*-4-methylthio-*m*-tolyl phosphorothioate; Bayer USA Inc.), by dipping the leaves for 30 seconds into 0.005% (v/v), 0.01% (v/v), 0.025% (v/v), 0.05% (v/v), or 0.15% (v/v) fenthion and 0.05% (v/v) L-77 Silwet (Union Carbide) dispersed in sterile water. The development of necrotic lesions was monitored visually for a period of 7 days after treatment. Five-and-a-half-week-old plants of *L. esculentum* cv. Moneymaker (*fen/fen*) and cv. Rio Grande-PtoR (*Fen/Fen*) were used as negative and positive controls, respectively. As a treatment control, all plants were exposed for 30 seconds to only 0.05% (v/v) L-77 Silwet dispersed in sterile water. The fenthion-exposed and control plants were grown under the environmental conditions described in section 3.1.

For determinative fenthion studies, six 5½-week-old plants of each accession

and cultivar listed in Table 3.5 were exposed to fenthion by dipping the leaves for 30 seconds into 0.005% (v/v) fenthion (Bayer USA Inc.) and 0.05% (v/v) L-77 Silwet (Union Carbide) dispersed in sterile water. Three leaves per plant were exposed to the fenthion/L-77 Silwet emulsion. The development of necrotic lesions was monitored visually for a period of 7 days after treatment. Two 5½-week-old plants of *L. esculentum* cv. Moneymaker (*fen/fen*) and cv. Rio Grande-PtoR (*Fen/Fen*) were used as negative and positive controls, respectively. As a treatment control, 6 plants of each *Brassica* accession and cultivar (Table 3.5) and 2 plants of each tomato cultivar were exposed for 30 seconds to only 0.05% (v/v) L-77 Silwet dispersed in sterile water. Three leaves per plant were exposed to the L-77 Silwet emulsion. The fenthion-exposed and control plants were grown under the environmental conditions described in section 3.1. Two replicates of this experiment were performed.

B. rapa L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were then chosen for a thorough analysis of the *Brassica* response to 0.005% fenthion exposure. These two cultivars were chosen since they showed considerably different average leaf necroses when exposed to 0.005% fenthion. Six 5½-week-old plants of *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were exposed to a 0.005% (v/v) fenthion (Bayer USA Inc.) and 0.05% (v/v) L-77 Silwet (Union Carbide). The plants were exposed to the fenthion/L-77 Silwet emulsion in the same manner as described above except every mature leaf on each plant was exposed to the fenthion/L-77 Silwet emulsion. As a treatment control, four 5½-week-old plants of *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were exposed to only 0.05% (v/v) L-77 Silwet. Similarly, every mature leaf on each control plant was exposed to the L-77 Silwet emulsion. Two plants of *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were left untreated and were not exposed to any chemical. These plants acted as standards that were compared to the fenthion/L-77 Silwet-exposed and L-77 Silwet-exposed plants. The development of necrotic lesions was monitored visually for a period of 7 days after treatment.

Fourteen 5½-week-old plants of *B. napus* L. cv. Excel, *B. napus* L. cv. Midas, *B. napus* L. cv. Quantum, *B. napus* L. cv. Westar, and *B. rapa* L. var. *oleifera* cv. R-

500 were exposed to malathion [diethyl (dimethoxyphosphinothioylthio)succinate; Green Cross, Mississauga, ON, Canada]. This insecticide was prepared as 0.05% (v/v) and 0.15% (v/v) solutions in 0.05% (v/v) L-77 Silwet (Union Carbide). Two leaves from each plant were exposed to the malathion solutions. That is, one leaf on each plant was exposed to 0.05% malathion and another leaf from each plant was exposed to 0.15% malathion. Six 5½-week-old plants of each cultivar listed above were used as treatment controls. Ten 5½-week-old plants of *L. esculentum* cv. Moneymaker (*fen/fen*) and cv. Rio Grande-PtoR (*Fen/Fen*) were used as negative and positive controls, respectively. Furthermore, four 5½-week-old *L. esculentum* cv. Moneymaker (*fen/fen*) and cv. Rio Grande-PtoR (*Fen/Fen*) were used as treatment controls. The methods of exposure and scoring were identical to the fenthion treatments.

3.9 PLANT BREEDING

Reciprocal crosses were made between *B. napus* L. cv. Polo and *B. napus* L. cv. Westar to obtain two F₁ populations. The two F₁ populations were selfed to obtain two F₂ populations. Reciprocal backcrosses were also performed with the two *B. napus* L. F₁ populations, *B. napus* L. cv. Polo, and *B. napus* L. cv. Westar to obtain eight, reciprocal backcross populations.

3.10 PLANT GENOMIC DNA ISOLATION

Leaves of individual plants were flash frozen in liquid nitrogen and then stored at -80°C. The frozen leaves were then lyophilized (VirTis Unitop 200 and Freezemobile 6 lyophilizer) at -20°C and subsequently ground to a fine powder in a commercial blender. The lyophilized leaf powder was stored in the dark at room temperature until DNA extraction. The protocol of Doyle and Doyle (1990) was used to isolate genomic DNA from 0.3 g of the lyophilized leaf powder with the exception that the cetyltrimethylammonium bromide (CTAB) isolation buffer contained 3% (w/v) CTAB and 0.4% β-mercaptoethanol. In addition, 15 mL of the CTAB isolation buffer was used in isolating genomic DNA from the 0.3 g of lyophilized leaf powder. The isolated DNA was then quantified using a Sequoia Turner 450 fluorometer. The

DNA samples were also ran out on 0.8%/0.5x TBE agarose gels to confirm their concentrations and to determine if there was any DNA degradation/shearing or RNA contamination.

3.11 POLYMERASE CHAIN REACTION

Three degenerate oligonucleotide primers (BKE-1, BKE-2, and BKE-3) were synthesized based on the deduced Pto amino acid sequence (Martin et al., 1993a; Table 3.6). BKE-1, BKE-2, and BKE-3 were designed from Pto kinase subdomain I, subdomain V, and subdomain XI, respectively (Martin et al., 1993a). In addition, these oligonucleotide primers were designed based on codon usage and minimal amino acid degeneracy in *B. napus* (Wada et al., 1992). Specifically, the primers were designed based on optimal methionine and tryptophan content and codons that have less than a 20% frequency in known *B. napus* genes were not used (Table 3.6).

BKE-1 and BKE-2 were sense (5') primers and were 64-fold and 32-fold degenerate, respectively, while BKE-3 was an antisense (3') primer and was 48-fold degenerate (Table 3.6). Primer combinations were as follows: I: primers BKE-1 plus BKE-3; and II: primers BKE-2 plus BKE-3. Sequences were amplified using PCR with primer combination I (BKE-1 and BKE-3), primer combination II (BKE-2 and BKE-3), primer BKE-1 alone, primer BKE-2 alone, and primer BKE-3 alone.

One and a half micrograms of genomic DNA (*B. napus* L. breeding lines 92-1817-1, 92-1819-1, and 92-1822-1) was amplified with 1 U *Taq* DNA polymerase (Boehringer Mannheim) in 100 μ L of 10 mM TrisHCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 0.2 mM of each dNTP, and 1.5 μ M of each primer. DNA amplification was performed on a RoboCycler 40 (Stratagene) temperature cycler and the cycle parameters were 3 min initial denaturation at 94°C; then 35 cycles each with 1 min denaturation at 94°C, 1 min annealing at the temperatures listed below, and 2 min elongation at 72°C. The annealing temperature of the reaction was decreased 1°C every second cycle from 50°C to 40°C, at which temperature 15 cycles were carried out (Don et al., 1991). The 35 cycles were followed by one elongation step at 72°C for 8 min. The amplification products were separated by electrophoresis in 1.2%/1x

TBE agarose gels and 4.5%/1x TBE polyacrylamide gels.

B. napus L. breeding line 92-1822-1 amplification products of the expected size (an approximate 570 bp doublet and 420 bp band) from primer combination II (BKE-2 and BKE-3) were eluted from the polyacrylamide gels according to Chory (1989). Fifty nanograms of these purified products were then used as template in a second PCR as described above except only primer combination II (BKE-2 and BKE-3) was used. The amplification products were separated by electrophoresis in 1.3%/1x TBE agarose gels.

3.12 CLONING OF PCR PRODUCTS AND SEQUENCE ANALYSIS

The approximate 540 bp band of the 570 bp doublet and the 420 bp band of the second PCR were purified from the 1.3% agarose gel using the GlassMax DNA Isolation Spin Cartridge System (GibcoBRL). The DNA concentrations of these eluted PCR products were determined by 1.3%/1x TBE agarose gel electrophoresis. The purified PCR products were cloned into the pGEM-T vector (Promega) by following the manufacturer's instructions. Thereafter, the cloned 540 bp and 420 bp PCR products were called *H-3* and *L-7*, respectively.

The pGEM-T vectors carrying the 540 bp (*H-3*) and 420 bp (*L-7*) PCR products were isolated from the *E. coli* JM109 transformants using Wizard Minipreps DNA Purification Systems (Promega) for use in manual sequencing. The pGEM-T vector carrying the 540 bp PCR product (*H-3*) was also isolated using the Qiagen Plasmid Maxi Kit with Qiagen-tip 500 columns for use in manual and automated sequencing.

The (+) DNA strand of *L-7* was sequenced by the dideoxy chain termination method (Sanger et al., 1977) using double-stranded DNA template, the T7 sequencing primer and a sequencing kit (Sequenase, United States Biochemicals). Double stranded sequencing of both DNA strands of *L-7* was obtained by PCR-based automated sequencing using T7 and pUC/M13 sequencing primers and the Applied Biosystems model 373A automated sequencer. Double stranded sequencing of both DNA strands of *H-3* using T7, pUC/M13, and SP6 sequencing primers was performed

by the dideoxy chain termination method (Sanger et al., 1977) using sequencing kits (Sequenase, United States Biochemicals and ³²P Sequencing Kit, Pharmacia Biotech). Additional sequence of *H-3* was obtained by PCR-based automated sequencing using T7 and SP6 sequencing primers and the Applied Biosystems model 373A and model 377 PRISM automated sequencers.

To acquire the entire sequence of both the sense and antisense strands of *H-3*, two additional sequencing primers were designed based on the *H-3* sequence information obtained from the T7, pUC/M13, and SP6 sequencing primers. These additional sequencing primers were designed using version 4.1 of the OLIGO Primer Analysis Software package (National Biosciences Inc., Plymouth, MN). The sense (5') sequencing primer sequence was 5'-GGAGTACTTCAGGAGACAGC-3' and the antisense (3') sequencing primer sequence was 5'-CCAAACCATTGCCCATTGAG-3'. Both sequencing primers were 20-mers. The sense sequencing primer represented the *H-3* DNA sequence from position 243 bp to 262 bp. The antisense sequencing primer represented the complementary DNA sequence of *H-3* from position 371 bp to 390 bp. The entire sequences of both the sense and antisense strands of *H-3* were obtained by PCR-based automated sequencing using the sense and antisense sequencing primers and the Applied Biosystems model 377 PRISM automated sequencer.

The nucleic acid sequence data obtained from the sequencing experiments were evaluated with the SeqAid II (version 3.5) computer software package (D.D. Rhoads and D.J. Roufa, Center for Basic Cancer Research, Kansas State University, Manhattan, KS, USA, 66506). The nucleic acid sequence was also translated into the complementary amino acid sequence in all six reading frames using the SeqAid II (version 3.5) computer software package. Version 1.4.11 of BLASTN (Altschul et al., 1990) was employed for nucleic acid similarity searches in the updated versions of the GenBank, EMBL, DDBJ, and PDB databases.

Version 1.4.11 of BLASTX (Altschul et al., 1990; Gish and States, 1993) was employed for amino acid similarity searches in the updated versions of the GenBank CDS, PDB, SwissProt, SPupdate, and PIR databases. The alignment of the Pto

(Martin et al., 1993a), pto (Jia et al., 1997), CrRLK1 (Schulze-Muth et al., 1996), 2642445 [GenBank accession number AC002391 and GenBank Identifier (GI) number 2642445], fen (Jia et al., 1997), and Fen (Martin et al., 1994) amino acid sequences to the H-3 amino acid sequence were made using the SeqAid II (version 3.5) software package, BLASTX search (Altschul et al., 1990; Gish and States, 1993) results, and eye. The identity and similarity between these proteins and H-3 was determined manually. For the percent similarity calculations, I used the structurally similar amino acid groupings of Hanks et al. (1988).

The serine/threonine protein kinase active-site signature in the H-3 polypeptide was identified using the PROSITE database (Bairoch, 1992; Bairoch et al., 1995), MacPattern sequence analysis software package (Fuchs, 1994), and Geneva University Hospital (University of Geneva, Geneva, Switzerland) ScanProsite analysis tool (on the ExPASy Molecular Biology Server; Appel et al., 1994). The serine/threonine protein kinase catalytic domain motifs in the H-3 polypeptide were identified according to Hanks et al. (1988); Hanks and Quinn (1991); Taylor et al. (1993); and Hanks and Hunter (1995). Finally, the amino acids that are invariant among all protein kinases were located in the H-3 polypeptide by using the information and consensus sequence presented by Hanks and Hunter (1995).

3.13 RFLP ANALYSIS

Genomic DNA of the plants listed in Table 3.7 was digested for 17 hours with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III according to the manufacturer's recommendations. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through 0.8% agarose/1x TBE gels. Using a Bio-Rad vacuum blotter (model 785), the resulting DNA fragments were vacuum blotted onto Hybond-N (Amersham) and GeneScreen Plus (DuPont) hybridization transfer membranes according to the method outlined in Appendix 8.1.

Hybridization membranes were initially wetted in 2x standard saline citrate (SSC). Pre-hybridization was carried out in 1% (w/v) dextran sulphate, 0.1% (w/v)

SDS, 0.2% (w/v) Ficoll (400), 0.2% (w/v) polyvinylpyrrolidone (360), 0.2% (w/v) BSA (fraction V), 0.6 M NaCl, 4 mM EDTA (pH 8), 0.12 M Tris-HCl (pH 8), 2.2 M tetra-sodium pyrophosphate containing 50 µg/mL herring testes DNA for 4 hours at 65°C. Following pre-hybridization, the membranes were hybridized with ³²P-labelled probes for 17 hours at 65°C in a fresh solution that was identical to the pre-hybridization solution except for the concentration of dextran sulphate which was changed to 10% (w/v). After hybridization, the membranes were washed twice with 2x SSC, 0.1% SDS for 20 min at 65°C and then washed once with 0.2x SSC, 0.1% SDS for 30 min at room temperature. Pre-hybridization, hybridization, and washing was also performed according to Hybond-N (Amersham) and GeneScreen *Plus* (DuPont; Section 1.6 Aqueous Hybridization Buffer for DNA) transfer and detection protocols. Low stringency pre-hybridization, hybridization, and washing was carried out at either 60°C or 55°C. For some low stringency washes, the membranes were only washed twice with 2x SSC, 0.1% SDS for 20 min at 55°C.

After washing, the membranes were wrapped in cling film and exposed to Kodak XAR-5 (X-OMAT AR) scientific imaging film at -80°C between two intensifying screens for the desired time. Sizes of the resulting hybridizing bands were calculated by reference to a lambda DNA *Hind*III digest and a lambda DNA *Bst*E II digest (New England Biolabs). Two replicate hybridizations were performed with each probe and each crop brassica (Table 3.7).

After developing the Kodak XAR-5 imaging film, some membranes were rewashed under high stringency conditions if they exhibited high background noise. Specifically, these membranes were washed twice with 0.1x SSC, 0.1% SDS for 30 min at 65°C. After the second high stringency washes the membranes were once again wrapped in cling film and exposed to Kodak XAR-5 (X-OMAT AR) scientific imaging film at -80°C between two intensifying screens for the desired time. If necessary, hybridization membranes were stripped according to Hybond-N (Amersham) and GeneScreen *Plus* (DuPont) stripping protocols.

3.14 OLIGOLABELLING OF *H-3*, *Fen*, *Pto*, AND *RPS2*

The probes used in the RFLP analyses (section 3.13) were *H-3*, *Fen* (Martin et al., 1994), *Pto* (Martin et al., 1993a), and *RPS2* (Bent et al., 1994; Mindrinos et al., 1994). The probe used in the *Fen* and *Pto* hybridization control experiment (section 3.15 below) was the *Fen* (Martin et al., 1994) gene. The vectors carrying the *H-3*, *Fen*, *Pto*, and *RPS2* inserts were isolated from the *E. coli* transformants using the Qiagen Plasmid Maxi Kit with Qiagen-tip 500 columns. The DNA was isolated from 200 mL of LB-Miller broth containing 100 µg/mL of ampicillin according to the midi/maxiplasmid/cosmid purification protocol contained in the Qiagen Plasmid Handbook (02/95; pp. 15-17). The OD₂₆₀ and OD₂₈₀ readings of the DNA samples were measured to determine DNA concentration and purity. The samples were also ran out on a 0.8%/0.5x TBE agarose gel to confirm the purity and determine the quality.

The pGEM-T (Promega) vector carrying *H-3* was digested with *NcoI* and *Sall*; the pCDNA II (Invitrogen) vectors carrying *Fen* and *Pto* were digested with *EcoRI*; and the pBluescript KS+ (Stratagene) vector carrying *RPS2* was digested with *Clal* and *EcoRV* according to the manufacturer's recommendations. These restriction digests released the *H-3*, *Fen*, *Pto*, and *RPS2* inserts from their respective vectors and these inserts were then separated from their vectors by electrophoresis through a 1.35%/1x TAE agarose gel. The separated inserts were then isolated from the agarose gel using the GlassMax DNA Isolation Spin Cartridge System (GibcoBRL) and the DNA concentrations of the inserts were determined by gel electrophoresis. Finally, the purified *H-3*, *Fen*, *Pto*, and *RPS2* inserts were labelled with ³²P-dCTP to high specific activity by oligolabelling using the ³²P-QuickPrime Kit (Pharmacia Biotech) and any unincorporated nucleotides were removed by using Sephadex G-50 NICK Columns (Pharmacia Biotech).

3.15 *Fen* AND *Pto* HYBRIDIZATION CONTROL EXPERIMENT

In an attempt to determine if *Fen* (Martin et al., 1994) or *Pto* (Martin et al., 1993a) could recognize the *H-3* sequence, the following experiment was conducted.

Approximately 10 genomic equivalents of the *H-3* insert was mixed with approximately 10 genomic equivalents of *H-3* in its linearized pGEM-T (Promega) vector and 6 μ g of sheared herring sperm DNA. The same procedure was carried out for the *Fen*, *Pto*, and *RPS2* inserts [please note *Fen* and *Pto* are carried in the pCDNA II (Invitrogen) vector and *RPS2* is carried in the pBluescript KS+ (Stratagene) vector]. The ten genomic equivalents for *H-3*, *Fen*, *Pto*, and *RPS2* were calculated by assuming they represented 3 kbp genes in *B. rapa* and by using the 500 megabase pair (Mbp)/1C (the unreplicated haploid genome of the species) nuclear DNA content value for *B. rapa* (Arumuganathan and Earle, 1991). The DNAs were then separated by electrophoresis through a 0.8% agarose/1x TBE gel.

Using a Bio-Rad vacuum blotter (model 785), the resulting DNA fragments were vacuum blotted onto GeneScreen *Plus* (DuPont) hybridization transfer membranes according to the method outlined in Appendix 8.1. Four membranes in total were prepared. Prior to pre-hybridization, the hybridization membranes were initially wetted in 2x SSC. Pre-hybridization and hybridization were performed as described in section 3.13 except they were carried out at 55°C. The membranes were hybridized with a ^{32} P-labelled *Fen* (Martin et al., 1994) probe and, after hybridization, the membranes were washed twice with 2x SSC, 0.1% SDS for 20 min at 55°C. After washing, the membranes were wrapped in cling film and exposed to Kodak XAR-5 (X-OMAT AR) scientific imaging film at -80°C between two intensifying screens for the desired time. Sizes of the resulting hybridizing bands were calculated by reference to a lambda DNA *Hind*III digest and a lambda DNA *Bst*E II digest (New England Biolabs).

After developing the Kodak XAR-5 imaging film, the membranes were rewashed at high stringency. Specifically, the membranes were washed twice with 0.1x SSC, 0.1% SDS for 30 min each at 65°C. After the second high stringency washes the membranes were once again wrapped in cling film and exposed to Kodak XAR-5 (X-OMAT AR) scientific imaging film at -80°C between two intensifying screens for the desired time.

3.16 BACTERIAL TRANSFORMATION OF *Fen*, *Pto*, AND *RPS2*

The *Fen* (Martin et al., 1994) and *Pto* (Martin et al., 1993a) genes carried in the pCDNA II vector (Invitrogen) and the *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) gene carried in the pBluescript KS+ (Stratagene) vector were transformed into *E. coli* strain DH5 α F' using a standard transformation protocol (Sambrook et al., 1989). The *Fen* (CD127) and *Pto* (CD186) cDNA clones were provided by Dr. S.D. Tanksley (Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY, USA). The *RPS2* cDNA clone was provided by Dr. B.J. Staskawicz (Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA).

Table 3.1. Plant Species, Accessions, Breeding Lines, and Cultivars Used in this Study.

Plant Species	Accessions/Breeding Lines/Cultivars	Source ^a
<i>Brassica napus</i> L.	cvs. Andor, Cascade, Cyclone, Delta, Elect, Excel, Hyola, Legend, Midas, Oro, Pivot, Polo, Profit, Quantum, Shaan 2233, Shiralee, Stellar, Topas, and Westar	Dr. G.R. Stringam
<i>B. napus</i> L.	breeding lines 87-6328-4B, 88-1409-K, 88-1426-K, 91-22220, 92-1817-1, 92-1819-1, 92-1822-1, and 92-20501	Dr. G.R. Stringam
<i>Brassica juncea</i>	cvs. Donskaya 4 and Cutlass	Dr. G.R. Stringam
<i>Brassica carinata</i>	accessions PI 21717 (Zambian) and PI 193460 (Ethiopian)	USDA
<i>Brassica rapa</i> L. var. <i>oleifera</i>	cvs. Eldorado, Goldrush, Horizon, Reward, R-500, Tobin, and Torch	Dr. G.R. Stringam
<i>B. rapa</i> var. <i>pekinensis</i> (bok choy)	Not Applicable	Apache Seeds Ltd.
<i>Brassica nigra</i>	accessions PISc ¹ , PI 271444 (Indian) ² , and PI 273642 (Ethiopian) ²	¹ Dr. G.R. Stringam ² USDA
<i>Brassica oleracea</i> var. <i>acephala</i> (kale)	cv. Dwarf Green Curled Scotch	Apache Seeds Ltd.
<i>B. oleracea</i> var. <i>capitata</i> (cabbage)	cv. Early Jersey Wakefield	Apache Seeds Ltd.
<i>B. oleracea</i> var. <i>italica</i> (broccoli)	cv. Italian Green Sprouting Calabrese	Apache Seeds Ltd.
<i>Lycopersicon</i> <i>esculentum</i>	cvs. Bonny Best ³ , Moneymaker ⁴ , and Rio Grande-PtoR ⁴	³ Apache Seeds Ltd. ⁴ Dr. G.B. Martin

^a Source information: Dr. G.R. Stringam, Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada; USDA, Ames Iowa USDA *Brassica* Germplasm Collection, USA; Apache Seeds Ltd., Edmonton, AB, Canada; and Dr. G.B. Martin, Department of Agronomy, Purdue University, West Lafayette, IN, USA.

Table 3.2. *Pseudomonas syringae* Pathovars, Isolates, and Strains Used in this Study.

<i>Pseudomonas</i> Species	Isolate/Strain	Source* and Reference
<i>P. syringae</i> pv. <i>maculicola</i>	84-59	Dr. D.A. Cuppels Wiebe and Campbell (1993)
<i>P. syringae</i> pv. <i>syringae</i>	Crak	Dr. P. Audy No reference
<i>P. syringae</i> pv. <i>syringae</i>	Crok	Dr. P. Audy No reference
<i>P. syringae</i> pv. <i>tomato</i> Race 0	DC3000	Dr. D.A. Cuppels Cuppels (1986)
<i>P. syringae</i> pv. <i>tomato</i> Race 0	DC3000(pLH12)	Dr. B.J. Staskawicz Whalen et al. (1991)
<i>P. syringae</i> pv. <i>tomato</i> Race 0	DC3000(pLH12 Ω)	Dr. B.J. Staskawicz Whalen et al. (1991)
<i>P. syringae</i> pv. <i>tomato</i> Race 1	T1	Dr. G.B. Martin Denny (1988a)
<i>P. syringae</i> pv. <i>tomato</i> Race 1	T1(pPtE6)	Dr. G.B. Martin Ronald et al. (1992)

* Source information: Dr. D.A. Cuppels, Agriculture and Agri-Food Canada, Pest Management Research Centre, London, ON, Canada; Dr. P. Audy, Agriculture and Agri-Food Canada, Fredericton Research Centre, Fredericton, NB, Canada; Dr. B.J. Staskawicz, Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA; and Dr. G.B. Martin, Department of Agronomy, Purdue University, West Lafayette, IN, USA.

Table 3.3. *Brassica napus* L. and *Brassica rapa* L. var. *oleifera* Breeding Lines and Cultivars Screened with Various *Pseudomonas syringae* Pathovars, Isolates, and Strains at an Inoculum Concentration of 1×10^8 cfu/mL.

<i>Brassica</i> Species	Breeding Lines/Cultivars	Source ^a
<i>B. napus</i> L.	cvs. Andor, Cascade, Cyclone, Delta, Elect, Excel, Hyola, Legend, Midas, Oro, Pivot, Polo, Profit, Quantum, Shaan 2233, Shiralee, Stellar, Topas, and Westar	Dr. G.R. Stringam
<i>B. napus</i> L. ^b	breeding lines 87-6328-4B, 88-1409-K, 88-1426-K, 91-22220, and 92-20501.	Dr. G.R. Stringam
<i>B. rapa</i> L. var. <i>oleifera</i>	cvs. Eldorado, Goldrush, Horizon, Reward, R-500, Tobin, and Torch	Dr. G.R. Stringam

^a Source information: Dr. G.R. Stringam, Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada.

^b Please note that the *B. napus* L. breeding lines were only inoculated with *P. syringae* pv. *syringae* isolates Crak and Crok. These *B. napus* L. breeding lines were not screened with any of the other *P. syringae* pathovars and strains (Table 3.2).

Table 3.4. *Brassica napus* L. and *Brassica rapa* L. var. *oleifera* Cultivars Screened with *Pseudomonas syringae* pv. *tomato* stains DC3000(pLH12), DC3000(pLH12 Ω), T1, and T1(pPtE6) Inocula of 5×10^6 cfu/mL.

<i>Brassica</i> Species	Cultivars	Source ^a
<i>B. napus</i> L.	Cascade, Excel, Midas, Polo, Quantum, Shaan 2233, Topas, and Westar	Dr. G.R. Stringam
<i>B. rapa</i> L. var. <i>oleifera</i>	Eldorado, Goldrush, Horizon, Reward, R-500, Tobin, and Torch	Dr. G.R. Stringam

^a Source information: Dr. G.R. Stringam, Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada.

Table 3.5. The Crop Brassicas Used in the Fenthion Assay.

<i>Brassica</i> Species	Accessions/Cultivars	Source ^a
<i>B. napus</i> L.	cvs. Excel, Midas, Polo, Quantum, Topas, and Westar	Dr. G.R. Stringam
<i>B. juncea</i>	cvs. Donskaya 4 and Cutlass	Dr. G.R. Stringam
<i>B. carinata</i>	accessions PI 21717 (Zambian) and PI 193460 (Ethiopian)	USDA
<i>B. rapa</i> L. var. <i>oleifera</i>	cvs. Eldorado, Goldrush, Horizon, Reward, R-500, Tobin, and Torch	Dr. G.R. Stringam
<i>B. rapa</i> var. <i>pekinensis</i> (bok choy)	Not Applicable	Apache Seeds Ltd.
<i>B. nigra</i>	accessions PI Sc ¹ , PI 271444 (Indian) ² , and PI 273642 (Ethiopian) ²	¹ Dr. G.R. Stringam ² USDA
<i>B. oleracea</i> var. <i>acephala</i> (kale)	cv. Dwarf Green Curled Scotch	Apache Seeds Ltd.
<i>B. oleracea</i> var. <i>capitata</i> (cabbage)	cv. Early Jersey Wakefield	Apache Seeds Ltd.
<i>B. oleracea</i> var. <i>italica</i> (broccoli)	cv. Italian Green Sprouting Calabrese	Apache Seeds Ltd.

^a Source information: Dr. G.R. Stringam, Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada; USDA, Ames Iowa USDA *Brassica* Germplasm Collection, USA; and Apache Seeds Ltd., Edmonton, AB, Canada.

Table 3.6. Degenerate Oligonucleotide Primers Based on the Deduced Amino Acid Sequence of Pto.

Pto Amino Acid Sequence ^a	Primer Name	Primer Sequence (5' to 3')
³⁹ NNFDHKF	BKE-1 (5')	AA(TC)AA(TC)TT(TC)GA(TC)CA(TC)AA(GA)TT
¹³⁴ MSMSWEQ	BKE-2 (5')	ATGTC(AGCT)ATGTC(AGCT)TGGGA(GA)CA
³⁰³ MGDVLWK	BKE-3 (3')	TTCCA(AGCT)AG(ACG)AC(GA)TC(AT)CCCAT

^a Reference: Martin et al. (1993a).

Table 3.7. The Crop Brassicas Used in the RFLP Analysis.

Brassica Species	Accessions/Cultivars	Source ^a
<i>B. napus</i> L.	cvs. Polo and Westar	Dr. G.R. Stringam
<i>B. rapa</i> L. var. <i>oleifera</i>	cv. Reward	Dr. G.R. Stringam
<i>B. rapa</i> var. <i>pekinensis</i> (bok choy)	Not Applicable	Apache Seeds Ltd.
<i>B. nigra</i>	accessions PI 271444 (Indian) and PI 273642 (Ethiopian)	USDA
<i>B. oleracea</i> var. <i>acephala</i> (kale)	cv. Dwarf Green Curled Scotch	Apache Seeds Ltd.
<i>B. oleracea</i> var. <i>italica</i> (broccoli)	cv. Italian Green Sprouting Calabrese	Apache Seeds Ltd.

^a Source information: Dr. G.R. Stringam, Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada; Apache Seeds Ltd., Edmonton, AB, Canada; and USDA, Ames Iowa USDA *Brassica* Germplasm Collection, USA.

4. RESULTS

4.1 REACTION OF CANOLA TO *Pseudomonas syringae*

Preliminary pathogenicity studies indicated that *P. syringae* pv. *syringae* isolates Crak and Crok (Table 3.2) were not pathogenic on the *B. napus* L. and *B. rapa* L. breeding lines and cultivars examined (Table 3.3). That is, neither necrosis nor chlorosis developed on the *P. syringae* pv. *syringae* inoculated plants.

P. syringae pv. *tomato* strains T1 (Denny, 1988a) and T1(pPtE6) (Ronald et al., 1992) were also not pathogenic on the vast majority of the *B. napus* L. cultivars examined (Tables 3.3 and 3.4). Specifically, these *P. syringae* pv. *tomato* strains were weakly virulent on *B. napus* L. cv. Cyclone, cv. Excel, cv. Midas, and cv. Shaan 2233. These phytopathogens were also weakly virulent on all of the *B. rapa* L. var. *oleifera* cultivars tested (Tables 3.3 and 3.4). Weakly virulent refers to the phytopathogen causing small, necrotic lesions on $\leq 10\%$ of the inoculated leaf surface. In addition, these *P. syringae* pv. *tomato* strains did not produce chlorosis on the inoculated plants. The virulence of *P. syringae* pv. *tomato* strains T1 and T1(pPtE6) remained constant irrespective of the inoculum concentrations employed (1×10^8 cfu/mL or 5×10^6 cfu/mL).

During the preliminary phytopathogen screenings, *P. syringae* pv. *maculicola* strain 84-59 (Wiebe and Campbell, 1993) was shown to be very weakly virulent to moderately virulent on the *B. napus* L. and *B. rapa* L. cultivars examined (Table 3.3). The infectivity of *P. syringae* pv. *maculicola* strain 84-59 is shown in Tables 4.1 and 4.2. Similarly, the preliminary phytopathogen screenings using bacterial inocula of 1×10^8 cfu/mL demonstrated that *P. syringae* pv. *tomato* strain DC3000(pLH12) (Whalen et al., 1991), *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω) (Whalen et al., 1991), and *Pst* DC3000 (Cuppels, 1986) were all very weakly virulent to moderately virulent on the *B. napus* L. and *B. rapa* L. cultivars that were screened (Tables 4.1 and 4.2). The pathogenicity studies using *P. syringae* pv. *tomato* strain DC3000(pLH12) and *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω) inocula of 5×10^6 cfu/mL produced the same results as the studies using bacterial inocula of 1×10^8 cfu/mL.

Pst DC3000 was then chosen for the determinative pathogenicity study (section 3.5.3) since it appeared to be the most virulent bacterial phytopathogen examined. *B. napus* L. cv. Polo and *B. napus* L. cv. Westar were chosen as the test plants for this extensive *Pst* DC3000 pathogenicity study since they exhibited the most divergent phenotypes. Specifically, *B. napus* L. cv. Polo seemed to be the most resistant (or at least the most tolerant) cultivar to *Pst* DC3000 and *B. napus* L. cv. Westar seemed to be the most severely infected cultivar by *Pst* DC3000 (Table 4.1).

In replicates one and two of the determinative pathogenicity study (section 3.5.3), *B. napus* L. cv. Polo exhibited a consistent phenotype after being inoculated with *Pst* DC3000. The inoculated *B. napus* L. cv. Polo plants remained symptomless up to three days after inoculation (Figure 4.1 A). By three days after inoculation, the *B. napus* L. cv. Polo leaves had small (less than 1 mm x 1 mm), necrotic flecks on them. These necrotic flecks covered $\leq 10\%$ of the leaf surfaces. By four days after inoculation, the necrotic flecks had slightly increased in number and size (1 mm x 1 mm) to cover $\leq 15\%$ of the leaf surfaces. However, there were a few inoculated *B. napus* L. cv. Polo leaves that did not show any response at four days after inoculation. The symptoms for the inoculated *B. napus* L. cv. Polo plants did not change for up to 12 days after inoculation. A representative *B. napus* L. cv. Polo leaf 8 days after *Pst* DC3000-inoculation is shown Figure 4.1 B. None of the *B. napus* L. cv. Polo control leaves showed any response for up to 12 days after treatment.

In replicate three of the determinative pathogenicity study (section 3.5.3), the progression of symptoms on *Pst* DC3000-inoculated, *B. napus* L. cv. Polo leaves was drastically different than observed in replicates one and two. All of the *B. napus* L. cv. Polo plants remained symptomless except for one plant, 1 day after inoculation. The leaves of this plant had approximately 10% of their surfaces covered with necrotic lesions (1 mm x 1 mm) that were surrounded by chlorotic tissue. By 2 days after inoculation, an additional 12 leaves exhibited small yellowish-green (chlorotic) streaks on their surfaces. Within 3 days of inoculation all of the *B. napus* L. cv. Polo leaves had yellowish-green spots (2 mm x 2 mm) that covered at least 20% of their surfaces.

These yellow/green spots turned into necrotic lesions by 4 days after inoculation.

The necrotic lesions began to coalesce and the tissue surrounding these lesions turned light green in colour within 5 days of inoculation. By eight days after inoculation, the number of lesions on all the leaves had increased in number and some of them had coalesced to form large (5 mm x 5 mm to 1 cm x 1 cm) necrotic areas. Most of the necrotic lesions were surrounded by varying degrees of chlorosis. All of the inoculated *B. napus* L. cv. Polo leaves had at least 30% of their surfaces covered with the disease symptoms but some of the inoculated leaves had approximately 70% of their surfaces covered with chlorotic and necrotic tissue. The symptoms for the inoculated *B. napus* L. cv. Polo plants remained fairly constant for up to 12 days after inoculation except the degree of chlorosis increased slightly and became more evident. One of the most severely diseased *B. napus* L. cv. Polo leaves at 8 days after inoculation with *Pst* DC3000 is shown in Figure 4.1 C. None of the *B. napus* L. cv. Polo control leaves showed any response within 12 days of treatment.

Unlike *B. napus* L. cv. Polo, *B. napus* L. cv. Westar showed a consistent phenotype in all three replicates of the *Pst* DC3000 determinative pathogenicity study (section 3.5.3). Additionally, the reaction of *B. napus* L. cv. Westar to the bacterial isolate (which later was proven to be *Pst* DC3000) in the pathogenicity study used to verify Koch's postulates (section 3.5.4) was also identical to the *B. napus* L. cv. Westar phenotypic response in the three replicates of the determinative pathogenicity study. After being inoculated with *Pst* DC3000, *B. napus* L. cv. Westar underwent a definite infection process and, consequently, developed severe disease symptoms.

All of the *B. napus* L. cv. Westar plants inoculated with *Pst* DC3000 remained symptomless up to 2 days after inoculation (Figure 4.2 B). Within 3 days of inoculation, all of the leaves had yellowish-green chlorotic patches covering 25-75% of the leaf surfaces. These chlorotic areas were 1 cm x 1 cm to 4 cm x 4 cm in size and contained necrotic flecks their centres. The *B. napus* L. cv. Westar leaves showed definite chlorosis and necrosis by 4 days after inoculation. The necrotic lesions were brown to greyish-tan in colour and ranged in size from flecks to 1 cm x 1 cm coalesced lesions. The necrosis and associated chlorosis covered 30-80% of the leaf

surfaces (Figure 4.2 C).

By six days after inoculation, the chlorotic areas had become definitely more yellow in appearance and the vast majority of the necrotic lesions had become light tan in colour. The necrotic lesions had also increased in size and some of them had coalesced into lesions as large as 2 cm x 4 cm. On average, 50% of each leaf was composed of chlorotic and necrotic tissue. The *Pst* DC3000-inoculated *B. napus* L. cv. Westar leaves showed essentially the same response at 8 days after inoculation. However, the chlorotic and necrotic tissue now constituted more than 50% of each leaf. Moreover, the necrotic lesions ranged in size from 2 mm x 2 mm to 2 cm x 4 cm and they were now composed of completely desiccated tissue. A representative *B. napus* L. cv. Westar leaf inoculated with *Pst* DC3000 8 days after inoculation is shown in Figure 4.2 D. The symptoms on the inoculated *B. napus* L. cv. Westar plants remained identical for up to 12 days after inoculation. None of the *B. napus* L. cv. Westar control leaves showed any response within 12 days of treatment (Figure 4.2 A).

4.2 *in planta* *Pst* DC3000 GROWTH

Bacterial growth in plant leaves was monitored to determine whether the lack of symptom development correlated with the restriction of bacterial multiplication. To quantify the growth of *Pst* DC3000 in the leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar, I performed three replicates of this bacterial growth curve experiment (Figure 4.3) during the determinative pathogenicity study (section 3.5.3). In all three replicates, I determined that there was no *Pst* DC3000 naturally present on the plants being examined prior to inoculation. Furthermore, approximately 3.8×10^3 cfu of *Pst* DC3000 per cm² of leaf tissue and 3.1×10^3 cfu of *Pst* DC3000 per cm² of leaf tissue were applied to *B. napus* L. cv. Polo and *B. napus* L. cv. Westar, respectively, during the inoculation procedure (Figure 4.3).

In the first replicate of this experiment I detected significant differences in the levels of *Pst* DC3000 in *B. napus* L. cv. Westar and *B. napus* L. cv. Polo. There was approximately 4-fold, 8-fold, 150-fold, and 40-fold more *Pst* DC3000 in *B. napus* L.

cv. Westar than in *B. napus* L. cv. Polo 2, 4, 6, and 8 days after inoculation, respectively (Figure 4.3 A). These data were encouraging since they supported the phenotypic responses of both *B. napus* L. cv. Westar and *B. napus* L. cv. Polo in this replicate of the determinative pathogenicity study (section 3.5.3). Furthermore, an approximate 200-fold difference in *in planta* bacterial levels is indicative of resistant and susceptible plant lines (Dr. D.A. Cuppels and Dr. G.B. Martin, personal communication).

Unfortunately, the next two replicates did not support the data obtained in replicate one. In the second replicate of the determinative pathogenicity study (section 3.5.3), there was only approximately 7-fold, 4-fold, and 3-fold more *Pst* DC3000 in *B. napus* L. cv. Westar than in *B. napus* L. cv. Polo 4, 6, and 8 days after inoculation, respectively (Figure 4.3 B). In the third, and final, replicate of this experiment, there was only 3-fold more *Pst* DC3000 in *B. napus* L. cv. Westar than in *B. napus* L. cv. Polo 6 days after inoculation (Figure 4.3 C).

When considering average values for all three replicates, there was only 5-fold (1.8×10^7 cfu/cm² versus 3.6×10^6 cfu/cm²) and 8-fold (2.5×10^6 cfu/cm² versus 3.2×10^5 cfu/cm²) more *Pst* DC3000 in *B. napus* L. cv. Westar than in *B. napus* L. cv. Polo at 4 and 6 days after inoculation, respectively (Figure 4.3 D). Consequently, when taking all three replicates of the determinative pathogenicity study (section 3.5.3) into account, *Pst* DC3000 has similar growth patterns in both *B. napus* L. cv. Westar and *B. napus* L. cv. Polo (Figure 4.3 D).

The differences between replicate one and the other two replicates was due to the variability of *Pst* DC3000 growth in *B. napus* L. cv. Polo (Figure 4.3). In replicate one (Figure 4.3 A) *Pst* DC3000 growth was significantly inhibited in *B. napus* L. cv. Polo 6 days after inoculation. However, *Pst* DC3000 growth in *B. napus* L. cv. Polo was not seriously inhibited during replicates 2 and 3 (Figure 4.3 B and C).

In contrast, the growth of *Pst* DC3000 in *B. napus* L. cv. Westar was relatively constant between all three replicates. None of the values obtained for the number of cfu/cm² of leaf tissue in *B. napus* L. cv. Westar 1, 2, 4, 6, and 8 days after inoculation were significantly different between the three replicates (Figure 4.3). During the study

used to verify Koch's postulates (section 3.5.4), I also determined the levels of the bacterial isolate (which was later proven to be *Pst* DC3000) in the leaves of *B. napus* L. cv. Westar at 8 days after inoculation. The bacterial isolate had multiplied to an average level of 2.0×10^6 cfu/cm² of leaf tissue which almost exactly matched the average value determined for *Pst* DC3000 growth in *B. napus* L. cv. Westar 8 days after inoculation (2.1×10^6 cfu/cm²; Figure 4.3 D). Accordingly, these experiments confirm the consistency of *Pst* DC3000 growth in *B. napus* L. cv. Westar.

Pst DC3000 multiplied approximately 6000-fold in *B. napus* L. cv. Westar over four days to reach a maximum leaf concentration of 1.8×10^7 cfu/cm² of leaf tissue while *Pst* DC3000 multiplied approximately 1000-fold in *B. napus* L. cv. Polo over four days to reach a maximum leaf concentration of 3.6×10^6 cfu/cm² of leaf tissue (Figure 4.3 D). By six days after inoculation the *Pst* DC3000 growth had significantly decreased in both *B. napus* L. cv. Westar and *B. napus* L. cv. Polo from the values seen at 4 days after inoculation (Figure 4.3 D). *Pst* DC3000 growth continued to decline in *B. napus* L. cv. Westar until 8 days after inoculation where it reached a level of 2.1×10^6 cfu/cm² (Figure 4.3 D). In contrast, the lowest level of *Pst* DC3000 growth in *B. napus* L. cv. Polo was 3.2×10^5 cfu/cm² of leaf tissue which was recorded 6 days after inoculation. By 8 days after inoculation, *Pst* DC3000 growth slightly increased in *B. napus* L. cv. Polo to a level of 5.4×10^5 cfu/cm² of leaf tissue (Figure 4.3 D). Therefore, by 8 days after inoculation there was 670-fold and 145-fold more *Pst* DC3000 in *B. napus* L. cv. Westar and *B. napus* L. cv. Polo, respectively, than was originally inoculated into these cultivars.

4.3 *Brassica napus* L. cv. POLO BY *Brassica napus* L. cv. WESTAR CROSSES

The reciprocal F₁, F₂, and backcross generations obtained from the breeding program between *B. napus* L. cv. Polo and *B. napus* L. cv. Westar could not be used in a segregation analysis study with *H-3*. The phenotypic variability of *B. napus* L. cv. Polo after inoculation with *Pst* DC3000 made it impossible to establish that *H-3* segregates with phenotypically defined *Pst* DC3000 resistance.

4.4 CHARACTERIZATION OF BACTERIA ISOLATED FROM DISEASED *Brassica napus* L. cv. WESTAR LEAF TISSUES

4.4.1 Morphological and cultural characteristics

Nine representative bacterial isolates were isolated from diseased *B. napus* L. cv. Westar leaf tissue as described in Materials and Methods (section 3.7.1). All 9 isolates were gram-negative rods that appeared to be identical in size and morphology. The KOH test (Suslow et al., 1982) also confirmed that all the isolates were gram-negative. A green to slightly yellow water-soluble fluorescent pigment was produced by all of the isolates when they were cultured on King's medium B agar. Finally, all of the isolates were unable to grow at 41°C. These characteristics of the bacterial isolates are recorded in Table 4.3 where they are compared to the known characteristics of *P. syringae* pv. *tomato*. The known *Pst* DC3000 control culture exhibited the same morphological and cultural characteristics as the 9 bacterial isolates (Table 4.3).

4.4.2 Physiological and biochemical reactions

All 9 isolates were shown to produce levan when grown on nutrient agar containing 5% sucrose and it was determined that all the isolates were catalase-positive. In addition, the isolates were negative for oxidase, arginine dihydrolase, nitrate reduction, and ice nucleation activity. All of the isolates also gave a negative potato soft rot reaction. Polypectate gel pitting occurred at pH 4.6, but not at pH 8.5, for all of the bacterial isolates. The 9 bacterial isolates also utilized *meso*-inositol, mannitol, and D(-)-tartrate as single carbon sources for growth. However, they were incapable of utilizing erythritol, DL-homoserine, DL-lactate, and sorbitol as single carbon sources for growth. All of these characteristics of the bacterial isolates are recorded in Table 4.3 where they are compared to the known characteristics of *P. syringae* pv. *tomato*. The known *Pst* DC3000 control culture exhibited the same physiological and biochemical characteristics as the 9 bacterial isolates (Table 4.3).

One of the most significant facts obtained from the nutritional profile analyses,

was that all 9 bacterial isolates did not utilize sorbitol (Table 4.3). This inability to utilize sorbitol as a sole carbon source for growth identified the 9 bacterial isolates as one of five *P. syringae* pv. *tomato* strains (Henderson et al., 1992; Cuppels and Ainsworth, 1995). In particular, *Pst* DC3000 is unable to utilize sorbitol (Table 4.3; Henderson et al., 1992; Cuppels and Ainsworth, 1995).

4.4.3 Hypersensitivity and pathogenicity tests

All 9 of the bacterial isolates were able to induce the HR on tobacco leaves within 24 hours of inoculation (Table 4.3). The known *Pst* DC3000 control culture induced this same reaction on tobacco leaves within 24 hours of inoculation (Table 4.3). Furthermore, the 9 bacterial isolates and the known *Pst* DC3000 control culture all produced typical bacterial speck symptoms on *L. esculentum* cv. Bonny Best plants within 5 days of inoculation (Table 4.3).

4.4.4 Coronatine production

Phytotoxin production appears to be a characteristic specific to the fluorescent pseudomonads and, specifically, it is a typical feature of the *P. syringae* pathovars (Gross and Cody, 1985). Phytotoxin production also appears to be species- or pathovar-specific (Mitchell, 1982). The phytotoxin coronatine is a terpenoid, chlorosis-inducing, secondary metabolite that is produced by *P. syringae* pathovars *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato* (Mitchell, 1982; Mitchell et al., 1983; Gross and Cody, 1985; Wiebe and Campbell, 1993; Cuppels and Ainsworth, 1995).

The tomato leaf bioassay for coronatine production revealed that the 9 bacterial isolates and the *Pst* DC3000 control culture were all strongly positive for the assay. The culture supernatants of these bacteria produced strong chlorotic zones that were ≥ 1 cm in diameter on tomato (*L. esculentum* cv. Bonny Best) leaves. Thus, the 9 bacterial isolates and the *Pst* DC3000 control culture were shown to produce the phytotoxin coronatine.

4.4.5 Plasmid profile analysis

All of the 9 bacterial isolates contained two large indigenous plasmids that were approximately 68 kbp and 72 kbp in size. The *Pst* DC3000 control culture also possessed these two plasmids. These plasmid profiles of the 9 bacterial isolates exactly match the known, published plasmid profile for *Pst* DC3000 (Cuppels and Ainsworth, 1995; Dr. D.A. Cuppels, personal communication).

4.4.6 Analyses prove that the isolates are *Pst* DC3000

These analyses definitively showed that all 9 bacterial isolates were, in fact, *Pst* DC3000. The first ten characteristics listed in Table 4.3 identified the bacterial isolates as *P. syringae*. The carbon utilization (Table 4.3), hypersensitivity (Table 4.3), pathogenicity (Table 4.3), and coronatine bioassay analyses distinguished the isolates as *P. syringae* pv. *tomato*. Finally, the plasmid profile analysis provided molecular evidence to specifically characterize the isolates as *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000).

4.5 REACTION OF THE CROP BRASSICAS TO FENTHION AND MALATHION

The development of necrotic lesions on various *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars in response to the organophosphorous insecticide, malathion, was discovered serendipitously. In an attempt to control an aphid outbreak on *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars being used as test plants in pathogen studies, malathion was applied to the plants. Three days after application of malathion, I noticed the presence of a few necrotic lesions on the leaves of some *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars. Since malathion is chemically related to fenthion (Worthing and Walker, 1987), the observation of malathion-induced necrotic lesions raised the possibility that a *Fen* (Martin et al., 1994) homolog might be present in *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars.

At 0.05% (v/v) and 0.15% (v/v) concentrations, malathion did not cause the

formation of necrotic lesions on *B. napus* L. cv. Excel, *B. napus* L. cv. Quantum, and *B. rapa* L. var. *oleifera* cv. R-500. Approximately 15% of the *B. napus* L. cv. Midas leaves and 7% *B. napus* L. cv. Westar leaves exposed to 0.15% (v/v) malathion showed less than 5% leaf necrosis. These symptoms on *B. napus* L. cv. Midas and cv. Westar appeared two days after exposure and did not change in appearance for 7 days after the treatment. None of the leaves of any of the aforementioned cultivars showed any response when exposed to 0.05% (v/v) malathion. In addition, neither the 0.15% nor the 0.05% malathion solutions caused any necrotic lesions on 5½-week-old plants of *L. esculentum* cv. Moneymaker (*fen/fen*) or *L. esculentum* cv. Rio Grande-PtoR (*Fen/Fen*). Finally, none of the control plants exposed to 0.05% L-77 Silwet alone developed necrotic lesions.

In an attempt to determine if the organophosphorous insecticide, fenthion, might have a more severe effect than malathion on the crop brassicas, 5½-week-old plants of the species listed in Table 3.5 were exposed to various fenthion concentrations. These studies indicated that all of the crop brassicas studied were sensitive to varying degrees to fenthion. That is, all of the crop brassicas tested developed necrotic lesions when exposed to fenthion. However, none of the L-77 Silwet-exposed control plant leaves developed necrotic lesions. These leaves remained symptomless and resembled untreated, healthy leaves (Figure 4.4 A and B).

The most severe responses occurred when the plants were exposed to 0.15% (v/v) fenthion and the mildest responses developed when the plants were exposed to 0.005% (v/v) fenthion. Unlike the malathion treated plants, necrotic lesions developed within 24 hours after exposure to fenthion and the symptoms increased to a maximum level by 4 to 5 days after fenthion treatment. Following the fourth or fifth day the symptoms remained constant. This progression of symptoms was observed on all of the crop brassicas studied and at all of the fenthion concentrations employed.

Although all the fenthion concentrations examined caused necrotic lesions on the crop brassicas, the 0.005% (v/v) fenthion treatment resulted in the greatest phenotypic differentiation between the various *Brassica* species, varieties, accessions, and cultivars examined. Five days after 0.005% (v/v) fenthion treatment there were

definite differences in the average leaf necroses between species, between varieties of the same species, and between accessions and cultivars within the same species (Tables 4.4 and 4.5).

However, these values were only the average response of the plants to 0.005% fenthion exposure (Tables 4.4 and 4.5). All of the accessions and cultivars of the various *Brassica* species and varieties tested showed extremely wide phenotypic ranges in response to fenthion exposure. Specifically, there was not a single accession or cultivar from any species examined that had a unique phenotypic range that did not overlap the other accessions or cultivars from that same species. These varying responses to fenthion exposure made it impossible to differentiate between the low response of one accession or cultivar and the high response of another accession or cultivar.

For instance, *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were chosen for a thorough analysis of the *Brassica* response to 0.005% fenthion exposure. These two cultivars were chosen since they had considerably different average leaf necroses (Table 4.4; Figure 4.4 C and D). After these two cultivars were examined in depth, it was determined that the phenotypic responses of *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) significantly overlapped at all time points beginning 2 days after treatment (Figure 4.5).

4.6 PCR AMPLIFICATION OF A *Brassica napus* L. *Pto* HOMOLOG

I used different degenerate primer combinations (designated I and II) to amplify genomic DNA of *B. napus* L. breeding lines 92-1817-1, 92-1819-1, and 92-1822-1. Identical PCR products were generated when amplifying either *B. napus* L. breeding line 92-1817-1, 92-1819-1, or 92-1822-1 genomic DNA with primer combination I (BKE-1 and BKE-3), primer combination II (BKE-2 and BKE-3), primer BKE-1 alone, primer BKE-2 alone, and primer BKE-3 alone. The PCR products amplified from *B. napus* L. breeding line 92-1822-1 genomic DNA using primer combination I (BKE-1 and BKE-3), primer combination II (BKE-2 and BKE-

3), primer BKE-1, primer BKE-2, and primer BKE-3 are shown in Figure 4.6.

Using primer combination I and primer combination II, several major products ranging in size from 1 kbp to 400 bp were amplified from all three of the *B. napus* L. breeding lines (Figure 4.6 - lanes 3 and 4). Numerous bands ranging in size from 2.5 kbp to 400 bp were also amplified from all three of the *B. napus* L. breeding lines using primer BKE-2 alone and primer BKE-3 alone (Figure 4.6 - lanes 6 and 7). No PCR products were generated when amplifying any of the *B. napus* L. breeding lines with primer BKE-1 alone (Figure 4.6 - lane 5).

Based on the DNA sequence of *Pto* (Martin et al., 1993a; GenBank accession number U02271), the expected size of the amplification product from *B. napus* L. using primer combination I was 813 bp and the expected size of the amplification product from *B. napus* using primer combination II was 528 bp. The closest product to 813 bp that was amplified with primer combination I was approximately 775 bp in length (Figure 4.6 - lane 3). However, because the PCR products amplified by primer combination I and primer BKE-3 alone were identical in appearance (Figure 4.6 - lanes 3 and 7), this product was not investigated further. Fortunately, there was a 570 bp doublet that was amplified from *B. napus* L. using primer combination II (Figure 4.6 - lane 4) and these products were in close agreement with the expected PCR product size of 528 bp.

Consequently, the 570 bp doublet amplified from *B. napus* L. breeding line 92-1822-1 using primer combination II was isolated and purified from the other products of this PCR. In addition, a *B. napus* L. breeding line 92-1822-1 amplification product of approximately 420 bp that was generated with primer combination II was also isolated and purified from the other products of this PCR. This 420 bp product was isolated and purified since it represented the major amplification product of this PCR (Figure 4.6 - lane 4).

The isolated 570 bp doublet and 420 bp fragment were then used as templates in a second PCR with primer combination II. This second PCR was performed to obtain a sufficient amount of the products for ligation into vectors and transformation into *E. coli*. The products of this second PCR are shown in Figure 4.7 (lanes 3 and

5).

As can be seen in Figure 4.7, this second PCR generated four additional products when the isolated 570 bp doublet was used as template and one additional product when the isolated 420 bp fragment was used as template. These results indicated that the 570 bp doublet and 420 bp fragment were actually heterogeneous PCR products or the 570 bp doublet and 420 bp fragment contained several BKE-2 or BKE-3 primer binding sites. Since low stringency PCR conditions were employed it is likely that mismatching occurred between the degenerate BKE-2 and BKE-3 primers and the templates. Therefore, the primers could have bound to several sites within the 570 bp doublet and 420 bp fragment and, consequently, amplified several different products.

The 540 bp band rather than the 600 bp band of the 570 bp doublet was randomly chosen for further analysis. Consequently, the 540 bp band of the 570 bp doublet was isolated and purified from the other amplification products of the second PCR. The 420 bp fragment was also isolated and purified from the other amplification product of the second PCR. The isolated 540 bp and 420 bp amplification products are shown in Figure 4.7 (lanes 4 and 6)

4.7 CLONING OF THE 540 bp AND 420 bp PCR PRODUCTS

The isolated 540 bp and 420 bp PCR products were then ligated into vectors and transformed into *E. coli*. Twelve recombinant clones for each PCR product were selected and restriction digests were performed on each chosen clone to release the PCR product inserts. All of the selected clones released appropriately sized inserts.

The inserts were not digested with restriction enzymes to identify RFLPs among the inserts. It was assumed that all the 420 bp and 540 bp inserts represented single and identical PCR products. Therefore, I cannot be assured that the 420 bp and 540 bp PCR products did not actually represent heterogeneous amplification products that were the same size.

A single recombinant clone carrying the 540 bp PCR product was chosen for further analysis. In addition, one recombinant clone of the 420 bp PCR product was

also chosen for further analysis. Thereafter, the 540 bp PCR product clone was called *H-3* and the 420 bp PCR product clone was called *L-7*.

4.8 SEQUENCE ANALYSIS OF *H-3* AND *L-7*

The nucleotide sequence of *L-7* was obtained for both the sense and antisense strands. No ORF was detected in any of the six amino acid sequence reading frames. That is, *L-7* could not be translated into a polypeptide uninterrupted by stop codons.

In addition, the *L-7* reading frame that resulted in a polypeptide sequence containing the *Pto*-derived BKE-2 and BKE-3 amino acid sequences, only shared 18% identity with *Pto* (Martin et al., 1993a). This complementary amino acid sequence of *L-7* also did not possess any of the amino acids that are invariant among protein kinases nor did it possess any of the consensus sequences indicative of serine/threonine protein kinase specificity (Hanks et al., 1988; Hanks and Quinn, 1991; Taylor et al., 1993; Hanks and Hunter, 1995). Furthermore, *L-7* was only 426 bp long and the corresponding sequence from *Pto* (Martin et al., 1993a) is 528 bp long. Therefore, the deduced amino acid sequence of *L-7* was 20% shorter than the corresponding region in *Pto* (Martin et al., 1993a).

The entire nucleotide sequence of *H-3* was determined and was found to constitute an ORF of 540 bp, as shown in Figure 4.8. The nucleotide sequence of *H-3* was then compared to known nucleotide sequences with a BLASTN search (Altschul et al., 1990). The top eight nucleic acid sequences returned are reported in Table 4.6.

Of these eight nucleic acid sequences, two were for unpublished *A. thaliana* genomic DNA sequences (Table 4.6). Of the published gene sequences with known functions, the BLASTN search (Altschul et al., 1990) revealed that *H-3* was most similar to the *L. pimpinellifolium* *Pto* disease resistance gene (Martin et al., 1993a; GenBank accession number U02271; Table 4.6). These two genes shared an E value of 1.2×10^{-55} [The E (expected) value refers to the number of similar matches expected by chance alone. The lower the E value, the more significant the match is.]. *H-3* has 63% nucleic acid identity with the corresponding region in *Pto* (Martin et al., 1993a).

and 58% nucleic acid identity with the corresponding region in *Fen* (Martin et al., 1994). This similarity between *H-3* and *Pto*; and *H-3* and *Fen* was not surprising as *Pto* and *Fen* are homologous genes that are contained in a *L. esculentum* gene cluster (Martin et al., 1994).

Using a BLASTX search (Altschul et al., 1990; Gish and States, 1993), the deduced amino acid sequence of the *H-3* ORF was compared to known protein sequences and proteins with the highest identity to *H-3* were identified. The most similar proteins to *H-3* were identified as the proteins encoded by six of the top eight nucleic acid sequences with DNA homology to *H-3* (Tables 4.7 and Table 4.6). The *H-3* amino acid sequence showed striking similarity with all of these proteins but it had the most similarity (E value = 4.5×10^{-74}) with the *L. pimpinellifolium* *Pto* serine/threonine protein kinase (Table 4.7).

The top 50 proteins identified with the BLASTX search (Altschul et al., 1990; Gish and States, 1993) were all plant proteins. Forty-three of these plant proteins were either serine/threonine protein kinases or receptor-like protein kinases. The next 50 most homologous proteins to *H-3* were composed of both plant and non-plant proteins and the vast majority of these were also protein kinases.

The entire deduced polypeptide of *H-3* was compared to the same region in the tomato *Pto* (Martin et al., 1993a), *Arabidopsis* 2642445, tomato *pto* (Jia et al., 1997), periwinkle CrRLK1 (Schulze-Muth et al., 1996), tomato *fen* (Jia et al., 1997), and tomato *Fen* (Martin et al., 1994) polypeptide sequences (Figure 4.9). It must be noted that 2642445 is the GI number for an unpublished, putative, 834 amino acid serine/threonine protein kinase from *A. thaliana* cv. Columbia. The GenBank accession number for 2642445 is AC002391.

Alignments to the deduced amino acid sequence of *H-3* were made by the SeqAid II (version 3.5) software package, BLASTX searches (Altschul et al., 1990; Gish and States, 1993), and eye. In order to optimize alignment, gaps were manually introduced into all of the sequences. By introducing gaps into the polypeptide sequences and maintaining a continuous polypeptide sequence for comparison (unlike the method used in the BLASTX searches), *H-3* was shown to share between 62-68%

identity and 76-82% similarity with all six proteins. The alignment of these proteins in decreasing order of percent identity and similarity with the deduced polypeptide of *H-3* is shown in Figure 4.9.

The *H-3* polypeptide contains all eight of the invariant amino acids found in protein kinases (Figure 4.10; Hanks and Hunter, 1995). These invariant amino acids are also found in the *Pto*, 2642445, *pto*, *CrRLK1*, *fen*, and *Fen* polypeptide sequences (Figure 4.9). The *H-3* polypeptide also possesses the serine/threonine protein kinase catalytic domains VIa through XI (Figure 4.10; Hanks et al., 1988; Hanks and Quinn, 1991; Taylor et al., 1993; Hanks and Hunter, 1995). In addition, the consensus sequence for serine/threonine kinases (Hanks and Hunter, 1995) is highly conserved in the *H-3* polypeptide and a serine/threonine protein kinase active-site signature was also detected in the *H-3* polypeptide (Bairoch, 1992; Appel et al., 1994; Fuchs, 1994; Bairoch et al., 1995). Both of these features are shown in Figure 4.10.

Importantly, the *H-3* polypeptide also contains a region that is very similar to the region in *Pto* that is required for the interaction with *AvrPto* (Figure 4.11; Scofield et al., 1996; Tang et al., 1996). If gaps are introduced into the *Pto* amino acid sequence to allow optimal alignment with the corresponding region in the *H-3* polypeptide, *H-3* shares 74% identity and 84% similarity with the *AvrPto*-interaction site in *Pto* as elucidated by Tang et al. (1996); and 71% identity and 88% similarity with the *AvrPto*-interaction site in *Pto* as elucidated by Scofield et al. (1996). The alignment of this region of the *H-3* polypeptide with the *AvrPto*-interaction site in *Pto* is shown in Figure 4.11.

4.9 RFLP ANALYSIS OF THE CROP BRASSICAS

Southern hybridization with ^{32}P -labelled *H-3* and *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) probes at 65°C gave positive signals in all of the crop brassicas analyzed (Figures 4.12 - 4.19). Two replicate hybridizations were performed with each probe and crop brassica (Table 3.7). Both hybridizations produced identical RFLP banding patterns. As can be seen in Figures 4.12 - 4.14 and 4.16 - 4.18, the *B. napus* L. cultivars (Polo and Westar) probed with *H-3* and *RPS2* appeared to have

similar RFLP banding patterns to the *B. rapa* varieties (*oleifera* and *pekinensis*) and *B. oleracea* varieties (*acephala* and *italica*) probed with *H-3* and *RPS2*. The *B. napus* L. RFLP banding patterns seemed to be composed of a mixture of bands from both *B. rapa* and *B. oleracea* (Figures 4.12 - 4.14 and 4.16 - 4.18).

However, there were some RFLP bands produced that were clearly distinct to *B. napus* L. and, therefore, were not observed in the *B. rapa* or *B. oleracea* varieties. Specifically, there was an approximate 5 kbp band generated by *Bam*HI digestion of *B. napus* L. cv. Westar, 6.6 kbp band generated by *Eco*RI digestion of *B. napus* L. cv. Westar, and 3.4 kbp band generated by *Hind*III digestion of *B. napus* L. cv. Polo that were unique to these cultivars when *B. napus* L. was probed with *H-3* (Figures 4.12 - 4.14). In addition, there was an approximate 3 kbp band generated by *Hind*III digestion of *B. napus* L. cv. Westar that was unique to this cultivar when it was probed with *RPS2* (Figures 4.16 - 4.18). All of the other RFLP bands seen in *B. napus* L. cv. Polo and *B. napus* L. cv. Westar appeared to be present in the *B. rapa* and *B. oleracea* varieties examined. None of the *B. nigra* (Table 3.7) RFLP bands generated by the *H-3* and *RPS2* probes corresponded to the *H-3* and *RPS2* RFLP bands seen in *B. napus* L. cv. Polo or *B. napus* L. cv. Westar (Figures 4.12, 4.15, 4.16, and 4.19).

Interestingly, neither *B. napus* L., *B. rapa*, nor *B. oleracea* DNA digested with *Bam*HI gave a positive signal when probed with *RPS2*. This particular restriction digest of these *Brassica* species resulted in only nonspecific background noise (Figures 4.16 - 4.18). Similarly, *B. nigra* DNA digested with *Eco*RI and *Hind*III and probed with *RPS2* also produced only nonspecific background noise (Figure 4.19). Moreover, *B. nigra* DNA digested with *Eco*RV and probed with *H-3* also did not give any positive signals (Figure 4.15).

Numerous polymorphisms were detected between both accessions/cultivars of each species examined (Table 3.7) when they were probed with *H-3* (Figures 4.12 - 4.15). However, only one polymorphism between both accessions/cultivars of each species examined was produced when they were probed with *RPS2* (Figures 4.16 -

4.19). Definite polymorphisms were also detected between the two plants of *B. rapa* L. var. *oleifera* cv. Reward, *B. oleracea* var. *acephala* (kale) cv. Dwarf Green Curled Scotch, and *B. oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese when they were probed with *H-3* (Figures 4.13 and 4.14). Likewise, a definite polymorphism was also detected between the two plants of *B. rapa* L. var. *oleifera* cv. Reward, *B. oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese, and *B. nigra* accession PI 271444 (Indian) when they were probed with *RPS2* (Figures 4.17 - 4.19). No polymorphisms were detected between the two plants of *B. napus* L. cv. Polo or *B. napus* L. cv. Westar when they were probed with either *H-3* or *RPS2* (Figures 4.12 and 4.16).

Unfortunately, ³²P-labelled *Fen* (Martin et al., 1994) and *Pto* (Martin et al., 1993a) probes did not detect any homologous sequences in the genomes of the analyzed crop brassicas (Table 3.7). That is, all of the Southern transfers probed with *Fen* and *Pto* showed nonspecific background noise with no positive signals even when hybridization was performed at 55°C. To determine if the washing conditions employed were too stringent and were removing bound probe, the membranes hybridized with *Fen* and *Pto* were only washed twice with 2x SSC, 0.1% SDS for 20 min at 55°C. Even when these very low stringency washes were used, no signal could be detected with the *Fen* and *Pto* probes.

A set of experiments was conducted to determine if the *Fen* and *Pto* probes were too divergent from *H-3* to detect the *Brassica* homolog. In these experiments, *B. napus* L., *B. rapa*, *B. oleracea*, and *B. nigra* genomic DNA RFLP blots probed with *H-3* (Figures 4.12 - 4.15) were left to decay until the signals were just faint bands. These blots were then re-probed with the *Fen* gene at 55°C. In addition, *B. napus* L., *B. rapa*, *B. oleracea*, and *B. nigra* genomic DNA RFLP blots probed with *RPS2* (Figures 4.16 - 4.19) were left to decay until the signals were just faint bands. These blots were then re-probed with the *Pto* gene at 55°C. None of these re-probed blots showed any additional bands after being probed with either *Fen* or *Pto*. Consequently, all of these blots were stripped and re-probed with *Fen* or *Pto* at 55°C. All of these

stripped, re-probed blots exhibited nonspecific background and none of them revealed any distinct signals.

A hybridization control experiment was also conducted to confirm that the *Fen* and *Pto* probes were too divergent from *H-3* to detect the *Brassica* homolog. In this experiment, 10 genomic equivalents of *H-3*, *Fen*, *Pto*, and *RPS2* were separated by electrophoresis through agarose gels and blotted onto hybridization membranes. The hybridization control blots were then placed in hybridization bottles containing *B. napus* L., *B. rapa*, *B. oleracea*, or *B. nigra* genomic DNA RFLP blots (see Table 3.7 for the specific cultivars and accessions). The membranes were then hybridized with ^{32}P -labelled *Fen* probes at 55°C.

All four of the hybridization control blots showed strong signals for the *Fen* and *Pto* genes (Figure 4.20). However, *H-3* and *RPS2* were not detected by the *Fen* probe on these control blots (Figure 4.20). Similarly, no signals were seen on the corresponding *B. napus* L., *B. rapa*, *B. oleracea*, and *B. nigra* genomic DNA RFLP blots hybridized with the *Fen* probes under the same conditions (ie. in the same hybridization bottles). After developing the films, the hybridization control membranes were rewashed at high stringency. Specifically, these membranes were washed twice with 0.1x SSC, 0.1% SDS for 30 min each at 65°C. These additional high stringency washes were unable to remove any of the *Fen* and *Pto* signal.

As a result, the melting temperature (T_m) of the DNA-DNA hybrid between the probe and the genomic fragment of interest was estimated for both the *Fen* and *Pto* probes using the following equation (Selden, 1989):

$$T_m = 81 + 16.6(\log_{10} C_i) + 0.4[\%(G+C)] - 0.6(\% \text{ formamide}) - 600/n - 1.5(\% \text{ mismatch})$$

In the equation, C_i refers to the salt concentration (monovalent ions) and n is the length of the hybrid in base pairs (Selden, 1989). To calculate the T_m of the *Fen* and *Pto* probes, 540 bp was used for the length of the hybrid (n). This value was chosen since the total length of *H-3* was 540 bp and the length of the entire *Pto* homolog in

the crop brassicas was unknown (ie. the complete gene was not cloned). Therefore, the %(G+C) and % mismatch were calculated based on the *H-3* sequence and the corresponding region in *Fen* or *Pto*.

Using these values and the above equation, the T_m of the DNA-DNA hybrid between the *Fen* probe and the *Brassica* genomic fragment of interest was determined to be 25°C; and the T_m of the DNA-DNA hybrid between the *Pto* probe and the *Brassica* genomic fragment of interest was determined to be 32°C. However, hybridization with *Fen* and *Pto* probes was not carried out at temperatures lower than 55°C. The hybridization temperature was not decreased because hybridization temperatures below 55°C are not stringent and, likely, would have permitted the binding of the *Fen* and *Pto* probes to nonhomologous sequences in the crop brassica genomes. In particular, using hybridization temperatures of 25°C and 32°C would have certainly allowed the *Fen* and *Pto* probes to hybridize to numerous nonhomologous sites in the *Brassica* genomes.

Table 4.1. Infectivity^a of Various *Pseudomonas syringae* Pathovars and Strains to *Brassica napus* L.

Cultivar	<i>Psm</i> 84-59 ^b	<i>Pst</i> DC3000(pLH12) ^c	<i>Pst</i> DC3000(pLH12 Ω) ^d	<i>Pst</i> DC3000 ^e
Andor	+/-	+/-	+/-	+/-
Cascade	+	+/-	+	+/-
Cyclone	++	+	+/-	+
Delta	+	+/-	+	+/-
Elect	+	+/-	+	+
Excel	+	+	++	++
Hyola	+	+/-	+/-	+
Legend	+/-	+	+	+
Midas	+	++	+	++
Oro	+/-	+/-	+/-	+
Pivot	+	+	++	++
Polo	+/-	+/-	+/-	+/-
Profit	+	+/-	+	+
Quantum	++	+/-	+/-	+
Shaan2233	+	+/-	+/-	+
Shiralee	+	+	+/-	+
Stellar	+	+	+	+
Topas	++	++	++	++
Westar	++	+	++	++

^a The infectivity ratings represent the degree of disease that the phytopathogens caused on 16 plants in two different experiments. Infectivity ratings: +/-, very weakly virulent (Does not produce necrotic lesions on some leaves and produces necrotic lesions covering $\leq 10\%$ of the leaf surface on the remaining leaves - no associated chlorosis.); +, weakly virulent (Produces necrotic lesions covering $\leq 15\%$ of the leaf surfaces. Lesions may be surrounded by chlorotic halos 1-2 mm in diameter.); and ++, moderately to highly virulent (Produces necrotic lesions surrounded by diffuse chlorosis covering $\leq 60\%$ of the leaf surfaces).

^b *Psm* 84-59 refers to *P. syringae* pv. *maculicola* strain 84-59 (Wiebe and Campbell, 1993).

^c *Pst* DC3000(pLH12) refers to *P. syringae* pv. *tomato* strain DC3000(pLH12) (Whalen et al., 1991).

^d *Pst* DC3000(pLH12 Ω) refers to *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω) (Whalen et al., 1991).

^e *Pst* DC3000 refers to *P. syringae* pv. *tomato* strain DC3000 (Cuppels, 1986).

Table 4.2. Infectivity^a of Various *Pseudomonas syringae* Pathovars and Strains to *Brassica rapa* L. var. *oleifera*.

Cultivar	<i>Psm</i> 84-59 ^b	<i>Pst</i> DC3000(pLH12) ^c	<i>Pst</i> DC3000(pLH12Ω) ^d	<i>Pst</i> DC3000 ^e
Eldorado	+/-	+	++	+
Goldrush	++	++	++	++
Horizon	+	+/-	+/-	+/-
Reward	+	+	+	+
R-500	+	++	++	++
Tobin	+	++	++	+
Torch	+	+	+/-	+

^a The infectivity ratings represent the degree of disease that the phytopathogens caused on 16 plants in two different experiments. Infectivity ratings: +/-, very weakly virulent (does not produce necrotic lesions on some leaves and produces necrotic lesions covering <10% of the leaf surface on the remaining leaves - no associated chlorosis); +, weakly virulent (produces necrotic lesions covering ≤15% of the leaf surfaces - no associated chlorosis); and ++, moderately to highly virulent (produces necrotic lesions covering ≤50% of the leaf surfaces. May also produce chlorosis in the tissue immediately surrounding the necrotic lesions).

^b *Psm* 84-59 refers to *P. syringae* pv. *maculicola* strain 84-59 (Wiebe and Campbell, 1993).

^c *Pst* DC3000(pLH12) refers to *P. syringae* pv. *tomato* strain DC3000(pLH12) (Whalen et al., 1991).

^d *Pst* DC3000(pLH12Ω) refers to *P. syringae* pv. *tomato* strain DC3000(pLH12Ω) (Whalen et al., 1991).

^e *Pst* DC3000 refers to *P. syringae* pv. *tomato* strain DC3000 (Cuppels, 1986).

Table 4.3. Characteristics of the Bacteria Isolated from Diseased *Brassica napus* L. cv. Westar Leaf Tissues Compared with the Characteristics of *Pseudomonas syringae* pv. *tomato*.

Characteristics	<i>P.s. pv. tomato</i> ^a	<i>Pst</i> DC3000 ^b	Isolated Bacteria
Gram stain	gram negative rods	gram negative rods	gram negative rods
KOH test	+	+	+
Fluorescein production	+	+	+
Growth at 41°C	-	-	-
Levan formation	+	+	+
Catalase	+	+	+
Oxidase	-	-	-
Arginine dihydrolase	-	-	-
Nitrate reduction	-	-	-
Ice nucleation activity	-	-	-
Potato soft rot	-	-	-
Pectate gel pitting ^c	4.6	4.6	4.6
Growth on:			
Erythritol	-	-	-
DL-Homoserine	-	-	-
meso-Inositol	+	+	+
DL-Lactate	-	-	-
Mannitol	+	+	+
Sorbitol	+/-	-	-
D(-)-Tartrate	+	+	+
HR on tobacco	+	+	+
Pathogenicity on tomato	+	+	+

^a *P.s. pv. tomato* refers to *P. syringae* pv. *tomato*. The characteristics of *P. syringae* pv. *tomato* are from the following sources: Schroth et al. (1983), Palleroni (1984), Hildebrand et al. (1988), Henderson et al. (1992), Schroth et al. (1992), Holt et al. (1994), and Cuppels and Ainsworth (1995).

^b *Pst* DC3000 refers to the pure *P. syringae* pv. *tomato* strain DC3000 (Cuppels, 1986) control culture that was subjected to all of the tests used to characterize the 9 bacterial isolates. These characteristics of *Pst* DC3000 exactly match the published characteristics for *Pst* DC3000 (see references above in ^a).

^c The pH at which pitting occurs.

Table 4.4. Response of the *Brassica* Elementary Diploid Species to 0.005% Fenthion Exposure.

<i>Brassica</i> species and variety	accessions/cultivars	Leaf necrosis (% area)	
		2 days	5 days
<i>B. rapa</i> L. var. <i>oleifera</i>	cv. Eldorado	5	10
	cv. Goldrush	5	10
	cv. Horizon	10	15
	cv. Reward	7	10
	cv. R-500	7	25
	cv. Tobin	10	15
	cv. Torch	5	10
<i>B. rapa</i> var. <i>pekinensis</i> (bok choy)	Not Applicable	25	35
<i>B. nigra</i>	accession PISc	15	20
	accession PI 271444 (Indian)	20	35
	accession PI 273642 (Ethiopian)	15	30
<i>B. oleracea</i> var. <i>acephala</i> (kale)	cv. Dwarf Green Curled Scotch	25	40
<i>B. oleracea</i> var. <i>capitata</i> (cabbage)	cv. Early Jersey Wakefield	10	30
<i>B. oleracea</i> var. <i>italica</i> (broccoli)	cv. Italian Green Sprouting Calabrese	20	40

Leaves of each plant were dipped into a solution of 0.005% fenthion and 0.05% L-77 Silwet dispersed in sterile water. Percentage of leaf area showing necrotic flecks was estimated visually at two time points after dipping. Values are the average response of 12 plants in two different experiments.

Table 4.5. Response of the *Brassica* Amphidiploid Species to 0.005% Fenthion Exposure.

<i>Brassica</i> species	accessions/cultivars	Leaf necrosis (% area)	
		2 days	5 days
<i>B. napus</i> L.	cv. Excel	5	15
	cv. Midas	25	40
	cv. Polo	30	45
	cv. Quantum	15	20
	cv. Topas	10	15
	cv. Westar	5	25
<i>B. juncea</i>	cv. Donskaya 4	20	40
	cv. Cutlass	10	25
<i>B. carinata</i>	accession PI 21717 (Zambian)	30	40
	accession PI 193460 (Ethiopian)	45	55

Leaves of each plant were dipped into a solution of 0.005% fenthion and 0.05% L-77 Silwet dispersed in sterile water. Percentage of leaf area showing necrotic flecks was estimated visually at two time points after dipping. Values are the average response of 12 plants in two different experiments.

Table 4.6. The Top Eight BLASTN Search Results for *H-3*.

Nucleic Acid Sequences with Homology to <i>H-3</i>	E value ^a
Unpublished <i>Arabidopsis thaliana</i> cv. Columbia genomic DNA sequence (GenBank accession number AB009056)	7.5x10 ⁻⁷⁷
<i>A. thaliana</i> cv. Columbia genomic DNA sequence (Kotani et al., 1997; GenBank accession number AB006705)	4.2x10 ⁻⁷⁶
Unpublished <i>A. thaliana</i> cv. Columbia genomic DNA sequence (GenBank accession number AC002391)	1.2x10 ⁻⁵⁶
<i>Lycopersicon pimpinellifolium</i> <i>Pto</i> gene (Martin et al., 1993a; GenBank accession number U02271)	1.2x10 ⁻⁵⁵
<i>Lycopersicon esculentum</i> <i>pto</i> gene (Jia et al., 1997; GenBank accession number U59316)	7.5x10 ⁻³⁴
<i>L. pimpinellifolium</i> <i>Fen</i> gene (Martin et al., 1994; GenBank accession number U13923)	3.4x10 ⁻²⁸
<i>L. esculentum</i> <i>fen</i> gene (Jia et al., 1997; GenBank accession number U59318)	3.4x10 ⁻²⁸
<i>Catharanthus roseus</i> <i>CrRLK1</i> gene (Schulze-Muth et al., 1996; GenBank accession number Z73295)	8.7x10 ⁻²⁴

^a The E (expected) value refers to the number of identical matches expected by chance alone. The lower the E value, the more significant the match is. The E values reported in this table were obtained by performing a BLASTN search (Altschul et al., 1990) using *H-3* as the query.

Table 4.7. The Top Six BLASTX Search Results for H-3.

Proteins with Homology to H-3	E value ^a
<i>Lycopersicon pimpinellifolium</i> Pto serine/threonine protein kinase (Martin et al., 1993a)	4.5×10^{-74}
Unpublished <i>Arabidopsis thaliana</i> putative serine/threonine protein kinase (GI number 2642445 and GenBank accession number AC002391)	4.7×10^{-71}
<i>Lycopersicon esculentum</i> pto serine/threonine protein kinase (Jia et al., 1997)	1.0×10^{-69}
<i>Catharanthus roseus</i> CrRLK1 receptor-like protein kinase (Schulze-Muth et al., 1996)	6.3×10^{-67}
<i>L. esculentum</i> fen serine/threonine protein kinase (Jia et al., 1997)	1.3×10^{-62}
<i>L. pimpinellifolium</i> Fen serine/threonine protein kinase (Martin et al., 1994)	1.0×10^{-60}

^a The E (expected) value refers to the number of similar matches expected by chance alone. The lower the E value, the more significant the match is. The E values reported in this table were obtained by performing a BLASTX search (Altschul et al., 1990; Gish and States, 1993) using H-3 as the query.

Figure 4.1. Reaction of *Brassica napus* L. cv. Polo Leaves to a 5×10^6 cfu/mL Inoculum of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000).

(A) A *B. napus* L. cv. Polo leaf inoculated with *Pst* DC3000 two days after inoculation. This photograph is representative of the leaves in replicate experiments one and two.

(B) A *B. napus* L. cv. Polo leaf inoculated with *Pst* DC3000 eight days after inoculation. This photograph is representative of the leaves in replicate experiments one and two.

(C) A *B. napus* L. cv. Polo leaf inoculated with *Pst* DC3000 eight days after inoculation. This photograph is representative of the most diseased leaves in replicate experiment three.



Figure 4.2. Reaction of *Brassica napus* L. cv. Westar Leaves to a 5×10^6 cfu/mL Inoculum of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000).

- (A) A *B. napus* L. cv. Westar control leaf two days after treatment.
- (B) A *B. napus* L. cv. Westar leaf inoculated with *Pst* DC3000 two days after inoculation.
- (C) A *B. napus* L. cv. Westar leaf inoculated with *Pst* DC3000 four days after inoculation.
- (D) A *B. napus* L. cv. Westar leaf inoculated with *Pst* DC3000 eight days after inoculation.

All photographs are of representative leaves taken at the indicated time points.



Figure 4.3. Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) in Leaves of *Brassica napus* L. cv. Polo and *Brassica napus* L. cv. Westar.

(A) Growth of *Pst* DC3000 in leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in replicate experiment one of the determinative pathogenicity study.

(B) Growth of *Pst* DC3000 in leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in replicate experiment two of the determinative pathogenicity study.

(C) Growth of *Pst* DC3000 in leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in replicate experiment three of the determinative pathogenicity study.

(D) Average growth of *Pst* DC3000 in leaves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in all three replicates of the determinative pathogenicity study.

Leaves of 3-week-old plants were inoculated with 5×10^6 cfu/mL of *Pst* DC3000 and bacterial populations were determined at the specified time points, as described in Materials and Methods. The day 0 time points represent the number of bacteria applied to the leaf surfaces during inoculation. In all four graphs, *B. napus* L. cv. Polo is represented by the open circle (○) data points and *B. napus* L. cv. Westar is represented by the filled circle (●) data points. For graphs (A), (B), and (C), values are the means of two samples, each consisting of three leaf disks from three different, infected leaves of one plant. For graph (D), values are the means of six samples, each consisting of three leaf disks from three different, infected leaves of one plant. Error bars equal one-half of the least significant difference at probability level 0.05. Means are significantly different where the error bars do not overlap (Martin et al., 1993a; Thilmony et al., 1995).

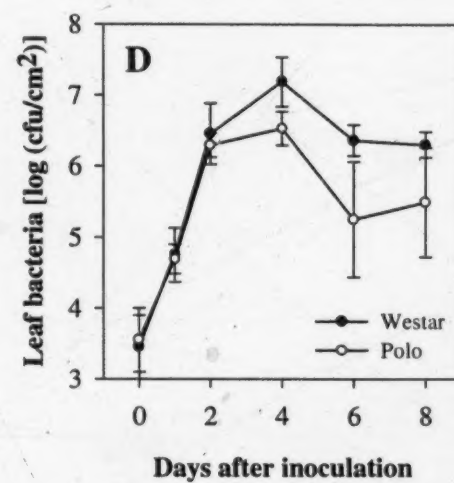
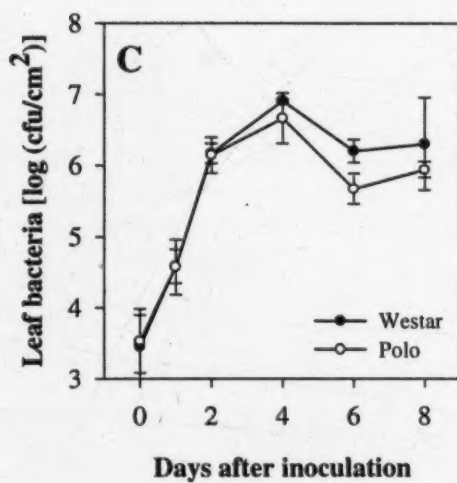
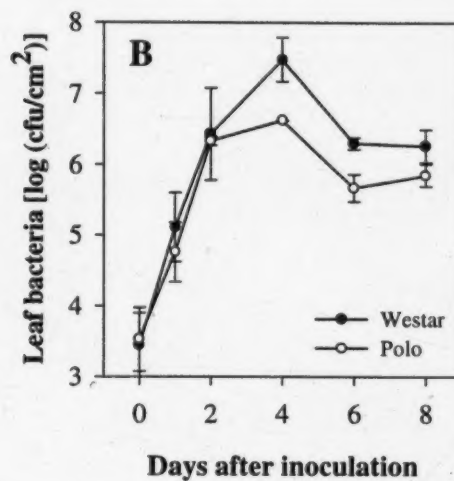
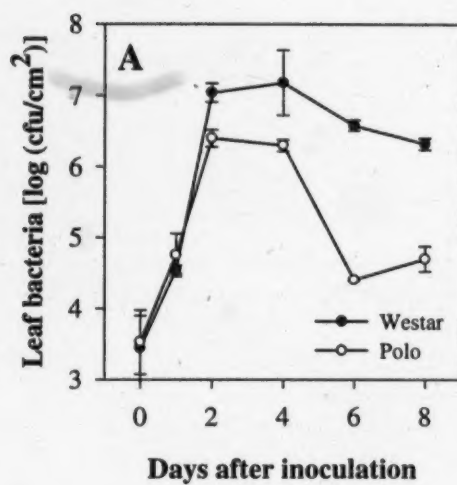


Figure 4.4. Symptoms on *Brassica rapa* L. var. *oleifera* cv. Reward and *Brassica rapa* var. *pekinensis* (bok choy) After Exposure to 0.05% L-77 Silwet and 0.005% Fenthion.

(A) A *B. rapa* L. var. *oleifera* cv. Reward control leaf immersed in 0.05% L-77 Silwet dispersed in sterile water. This photograph was taken two days after treatment and is representative of all *B. rapa* L. var. *oleifera* cv. Reward control leaves.

(B) A *B. rapa* var. *pekinensis* (bok choy) control leaf immersed in 0.05% L-77 Silwet dispersed in sterile water. This photograph was taken two days after treatment and is representative of all *B. rapa* var. *pekinensis* (bok choy) control leaves.

(C) A *B. rapa* L. var. *oleifera* cv. Reward leaf immersed in 0.005% fenthion and 0.05% L-77 Silwet dispersed in sterile water. This photograph was taken five days after treatment and represents the average response of *B. rapa* L. var. *oleifera* cv. Reward to 0.005% fenthion exposure.

(D) A *B. rapa* var. *pekinensis* (bok choy) control leaf immersed in 0.005% fenthion and 0.05% L-77 Silwet dispersed in sterile water. This photograph was taken five days after treatment and represents the average response of *B. rapa* var. *pekinensis* (bok choy) to 0.005% fenthion exposure.



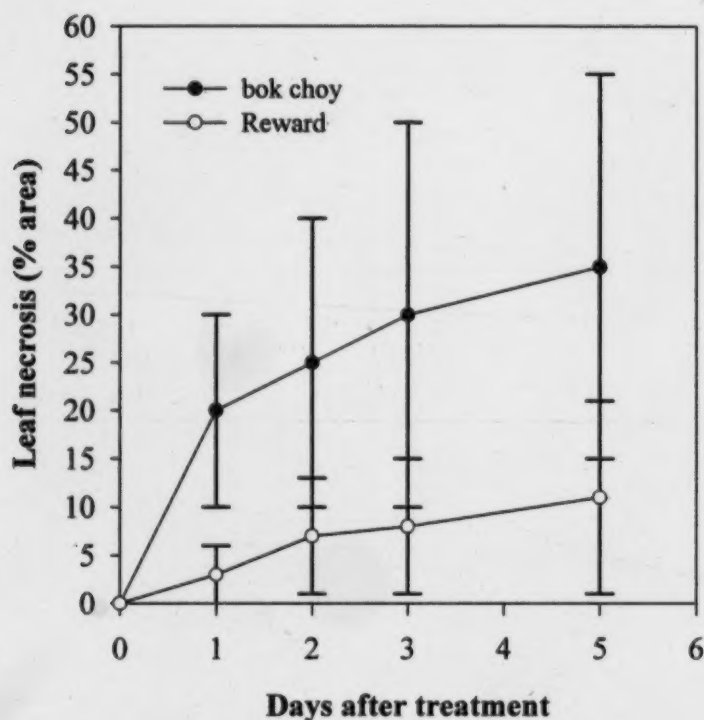


Figure 4.5. Reactions to 0.005% Fenthion Exposure in Leaves from *Brassica rapa* L. var. *oleifera* cv. Reward and *Brassica rapa* var. *pekinensis* (bok choy).

Leaves of each plant were dipped into a solution of 0.005% fenthion and 0.05% L-77 Silwet dispersed in sterile water. Percentage of the leaf area showing necrosis was estimated visually at four time points after dipping (1, 2, 3, and 5 days). Values are the average response of eighteen plants in three different experiments. The open circle (○) data points represent *B. rapa* L. var. *oleifera* cv. Reward and the closed circle (●) data points represent *B. rapa* var. *pekinensis* (bok choy). The vertical bars represent the high and low values of the phenotypic ranges of the plants. They do not represent error bars.

Figure 4.6. PCR Amplification Products of *Brassica napus* L. breeding line 92-1822-1 Genomic DNA using Primer Combination I (BKE-1 and BKE-3), Primer Combination II (BKE-2 and BKE-3), Primer BKE-1, Primer BKE-2, and Primer BKE-3.

Lanes 1 and 9 are "100 bp ladders" (Pharmacia Biotech) that were used as DNA size markers. Lanes 2 and 8 are empty lanes. Lanes 3 through 7 contain *B. napus* L. breeding line 92-1822-1 genomic DNA amplified with primer combination I (lane 3), primer combination II (lane 4), primer BKE-1 (lane 5), primer BKE-2 (lane 6), and primer BKE-3 (lane 7), respectively. The amplification products were separated on a 1.2% agarose gel at 150V (constant voltage) for one hour and 30 minutes. The arrows indicate the PCR products amplified with primer combination II that were isolated and purified from the other PCR products for use in further studies. Identical PCR products were generated when amplifying either *B. napus* L. breeding line 92-1817-1 or 92-1819-1 genomic DNA with primer combination I, primer combination II, primer BKE-1, primer BKE-2, and primer BKE-3.

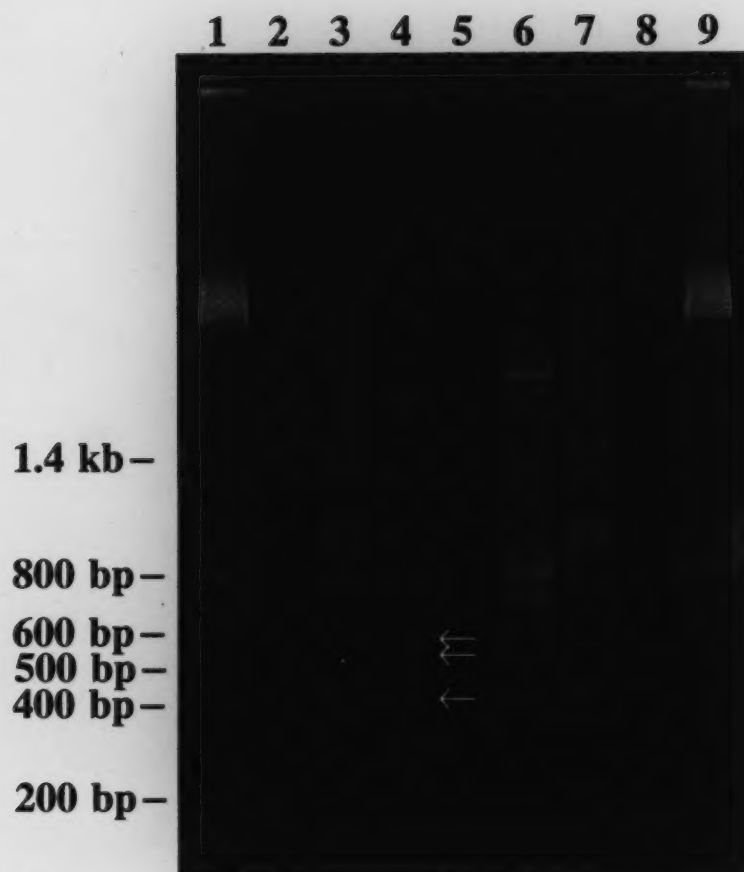
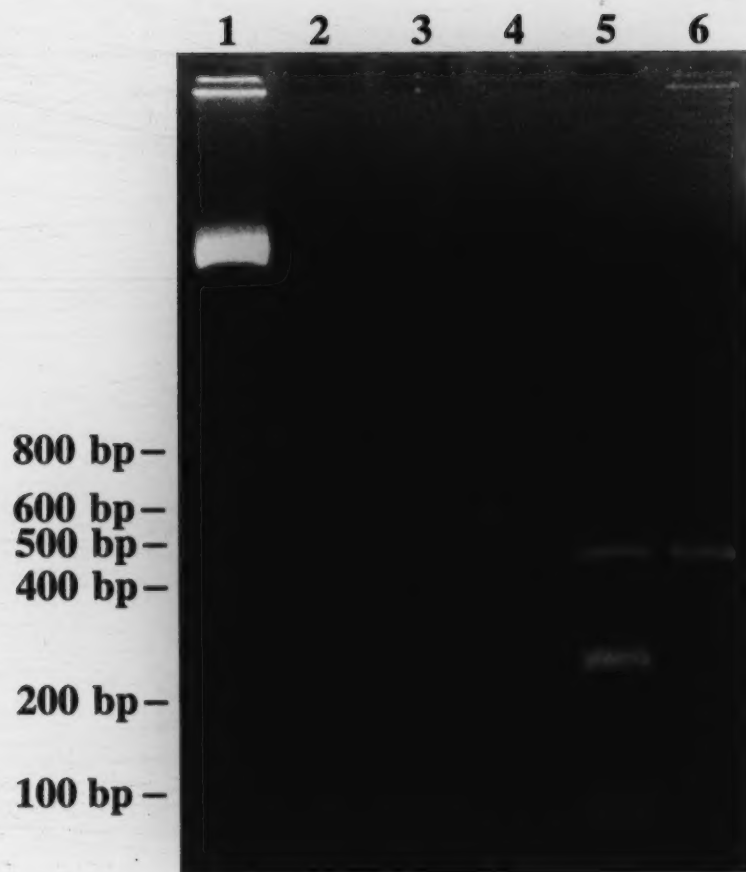


Figure 4.7. Purified PCR Products of *B. napus* L. breeding line 92-1822-1 Genomic DNA Amplified with Primer Combination II (BKE-2 and BKE-3).

B. napus L. breeding line 92-1822-1 amplification products of the expected size (an approximate 550 bp doublet and 420 bp fragment) generated by primer combination II were eluted from 4.5% polyacrylamide gels as described in Materials and Methods. These purified products and the PCR products amplified from *B. napus* breeding line 92-1822-1 genomic DNA using primer combination II, primer BKE-2, and primer BKE-3 were then run out on a 1.3% agarose gel at 100V (constant voltage) for one hour. Lane 1 is a "100 bp ladder" (Pharmacia Biotech) that was used as a DNA size marker. Lane 2 is an empty lane. Lanes 3 and 4 are the purified 550 bp doublet and 420 bp fragment, respectively. Lanes 5 through 7 contain *B. napus* L. breeding line 92-1822-1 genomic DNA amplified with primer combination II, primer BKE-2, and primer BKE-3, respectively.



<u>ATGTCTATGTCGTCGGGAGCAAAGACTCGAGTTTGCATCGGTGCAGCGAGG</u>	51
M S M S W E Q R L E V C I G A A R	17
GGGTTGCATTACCTGCACACCGGCGGGGCATCGGAGGGCATCATAACCGC	102
G L H Y L H T G G A S E G I I H R	34
GACGTGAAGACTACTAACATCCTACTAGACGAGAATCTAGTGGCGAAGGTC	153
D V K T T N I L L D E N L V A K V	51
CGGATTTTGGTTTATCCAAAACCGGACCTTCGCTTGATCAAACCTCATGTG	204
A D F G L S K T G P S L D Q T H V	68
AGCACTGCGGTTAAAGGAAGCTTTGGGTATCTCGACCCGGAGTACTTCAGG	255
S T A V K G S F G Y L D P E Y F R	85
AGACAGCAGTTAACCGAGAAGTCAGACGTGTACTCGTTCGGGGTTGTTCTG	306
R Q Q L T E K S D V Y S F G V V L	102
ATGGAAGTGCTCTGTTGTAGACCGGCTTTAAACCCGGTTTTGCCGAGGGAG	357
M E V L C C R P A L N P V L P R E	119
CAAGTGAACATAGCTGAATGGGCAATGGTTTGGCAGAAGAAGGTTCTTCTT	408
Q V N I A E W A M V W Q K K G L L	136
GATCAGATCATGGACGGTAACTTAACCGGGAAAGTGAACACTGCGTCTTTG	459
D Q I M D G N L T G K V N T A S L	153
AAGAAATTTGGTGAAACGGCTGAGAAGTGTTTAGAGGAGTATGGAGTGGAT	510
K K F G E T A E K C L E E Y G V D	170
<u>AGGCCGTCGATGGGAGACGTCCTCTGGAAA</u>	540
R P S M G D V L W K	180

Figure 4.8. DNA Sequence and Derived Amino Acid Sequence of *H-3*.

The amino acid sequence is presented in single letter code below the nucleotide sequence starting with the first base pair (the +1 frame). The PCR primer sites used to amplify this product are underlined.

Figure 4.9. Alignment of the Pto, pto, CrRLK1, 2642445, fen, and Fen Amino Acid Sequences to the H-3 Amino Acid Sequence.

The entire H-3 amino acid sequence was compared to the same region in Pto (Martin et al., 1993a), pto (Jia et al., 1997), CrRLK1 (Schulze-Muth et al., 1996), 2642445, fen (Jia et al., 1997), and Fen (Martin et al., 1994). The amino acid sequences shown are residues 1-180 of H-3, 134-309 of Pto, 128-299 of pto, 565-741 of CrRLK1, 580-757 of 2642445, 134-303 of fen, and 133-301 of Fen. Alignments to the deduced amino acid sequence of H-3 were made by using the SeqAid II (version 3.5) software package, BLASTX searches (Altschul et al., 1990; Gish and States, 1993) and eye. Each amino acid sequence (Pto, pto, CrRLK1, 2642445, fen, and Fen) was compared only to the H-3 sequence and not to any other sequences. The single letter amino acid code is used and gaps, represented by dashes, were introduced into the sequences to optimize alignment. Dots represent identical amino acid residues that are found between H-3 and the other proteins. Conserved residues found between the H-3 sequence and the other amino acid sequences are shown as white letters in black boxes. Structurally similar groupings used for this purpose are defined as follows: nonpolar chain R groups (M=L=I=V=C); aromatic or ring-containing R groups (F=Y=W=H); small R groups with near neutral polarity (A=G=S=T=P); acidic and uncharged polar R groups (D=E=N=Q); and basic polar R groups (K=R=H) (Hanks et al., 1988). 2642445 is the Genbank Identifier (GI) number for an unpublished, putative, 834 amino acid serine/threonine protein kinase from *Arabidopsis thaliana* cv. Columbia. The GenBank accession number for 2642445 is AC002391. It must be noted that the first and last seven amino acids of the H-3 polypeptide were contributed by the BKE-2 and BKE-3 degenerate PCR primer sequences and, therefore, may not correspond to the genomic DNA sequence exactly.

H-3	MSMSWEQRLEVCIGAARGHLHYLHTGGASEG-IIHRDVKTTNILLDENLVAK	50
Pto	R-....I.....F..	179
pto	N-....F..	172
CrRLK1	-K N.RK. MKR- ..	612
2642445	-.K. D. A. HN	628
fen	N-....C.....F..	179
Fen	KN-....C.....F..	177
H-3	VADFGLSKTGP-SLDQTHVSTAVKGSFGYLDPEYFRROQLTEKSDVYSFGV	100
Pto	...K.-E....V...L.....I GR	229
pto	...R-Q.Y.---D.....I GR	219
CrRLK1	DHF.....LTM.K.....	663
2642445	-IH-NQ...IN...LQTHK.....	677
fen	...M.-E..L...V...NI...A...ALWG.....	229
Fen	...M.-E....V...NI...A...ALWG.....	227
H-3	VLMEVLCCRPA LNPVLPREQVNIAEWAMVWQKKGLLDQIMDGNLTGKVNT	150
Pto	..F....A...VQS....M.....ESHNN.Q...AD..IR	279
pto	..F....A...VQS....M.....ESHNN.Q...AD..IR	269
CrRLK1	...TG..V...SK...M...V...KCS...VNE.R	711
2642445	...FA...Y...V.FCKS..T...S.I.Q	727
fen	..F....A...-Y...SDD-ET..M.Q...A...R	273
Fen	..F....A...R-...LDD-ET..M.Q...ETLAA..R	271
H-3	ASLKKFGETAEKCLEEYGVD RPSMGDVLWK	180
Pto	E...V...ALS E.....	309
pto	E...V...ALS E.....	299
CrRLK1	E..I..Q.....A.R.....VD..N	741
2642445	N.....M.I....K...D...R...D	757
fen	E...M.....M...APS K.....	303
Fen	E...M.....I...APS K.....	301

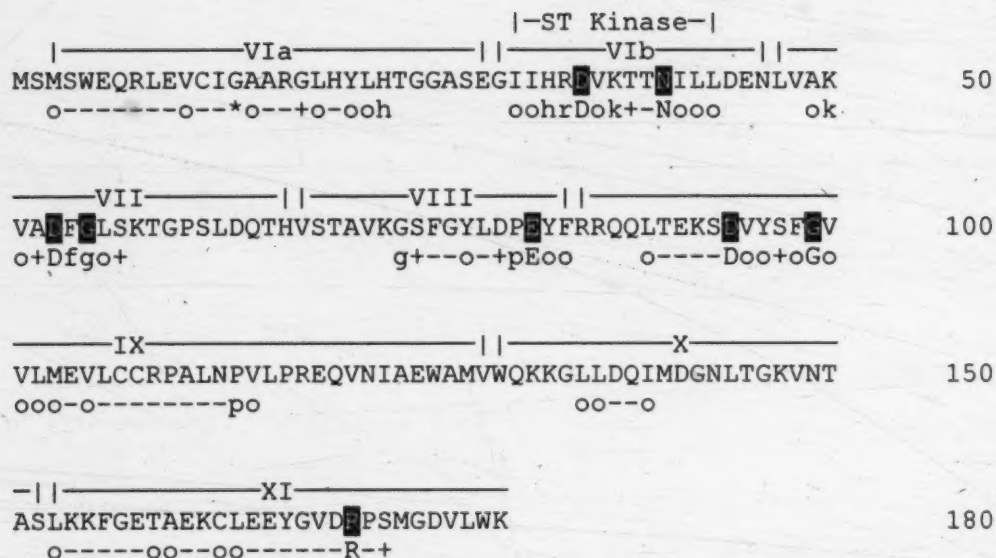


Figure 4.10. Serine/Threonine Protein Kinase Catalytic Domains Present in the Deduced Amino Acid Sequence of *H-3*.

The amino acid sequence is presented in single letter code and the amino acids that are invariant among all protein kinases (Hanks and Hunter, 1995) are shown as white letters in black boxes. The catalytic subdomains conserved in serine/threonine kinases (Hanks and Hunter, 1995) are indicated by the symbols VIa through XI above the *H-3* polypeptide sequence. Below the deduced amino acid sequence of *H-3* is the consensus sequence for serine/threonine kinases (Hanks and Hunter, 1995). The symbols used in the consensus sequence are as follows: upper case letters represent invariant residues, lowercase letters represent nearly invariant residues, o represents positions conserving nonpolar residues, * represents positions conserving polar residues, + represents positions conserving small residues with near neutral polarity, and - represents any residue (Hanks and Hunter, 1995). 'ST kinase' represents the serine/threonine protein kinase signature (Bairoch, 1992; Appel et al., 1994; Fuchs, 1994; Bairoch et al., 1995).

Pto	SDLPTMSMSWEQRLEICIGAARGLHYLHTR-A---IIHRDVKSINILLDE	174
H-3	*****.....[G].....GG.SEG.....TT.....	46
Pto	NFVPKITDFGISKKGTELDQTHLSTVVKGTGLGYIDPEYFIKGRLTEKSDV	224
H-3	.L.[L].[V].....[L].T.[S].....[L].A...[S].[F].....R[Q]Q.....	95

Figure 4.11. Alignment of the AvrPto-Interaction Site in Pto with the Same Region in the H-3 polypeptide.

The portion of the serine/threonine protein kinase domain which confers AvrPto specificity (Scofield et al., 1996; Tang et al., 1996) to the tomato R protein, Pto (Martin et al., 1993a), was compared to the same region in the H-3 polypeptide. The amino acid sequences shown are residues 129-224 of Pto and residues 1-95 of H-3. The Pto sequence listed in the figure is the AvrPto-interaction site elucidated by Tang et al. (1996) while the overlined sequence of Pto indicates the AvrPto-interaction site defined by Scofield et al. (1996). The single letter amino acid code is used and gaps, represented by dashes, were introduced into the Pto sequence to optimize alignment with H-3. Dots represent identical amino acid residues that are found between Pto and H-3. Conserved residues found between Pto and H-3 are shown as white letters in black boxes. Structurally similar groupings used for this purpose are defined as follows: nonpolar chain R groups (M=L=I=V=C); aromatic or ring-containing R groups (F=Y=W=H); small R groups with near neutral polarity (A=G=S=T=P); acidic and uncharged polar R groups (D=E=N=Q); and basic polar R groups (K=R=H) (Hanks et al., 1988). The asterisks at the beginning of the H-3 sequence represent amino acids that are not present in the H-3 polypeptide.

Figure 4.12. Southern Hybridization Analysis of *H-3* in *Brassica napus* L. cv. Polo and *Brassica napus* L. cv. Westar.

Genomic DNA from *B. napus* L. cv. Polo and *B. napus* L. cv. Westar was isolated from leaves. Two different plants from both *B. napus* L. cv. Polo and *B. napus* L. cv. Westar were used to isolate two separate DNA samples for each cultivar. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *H-3* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested *B. napus* L. cv. Westar genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested *B. napus* L. cv. Polo genomic DNA.

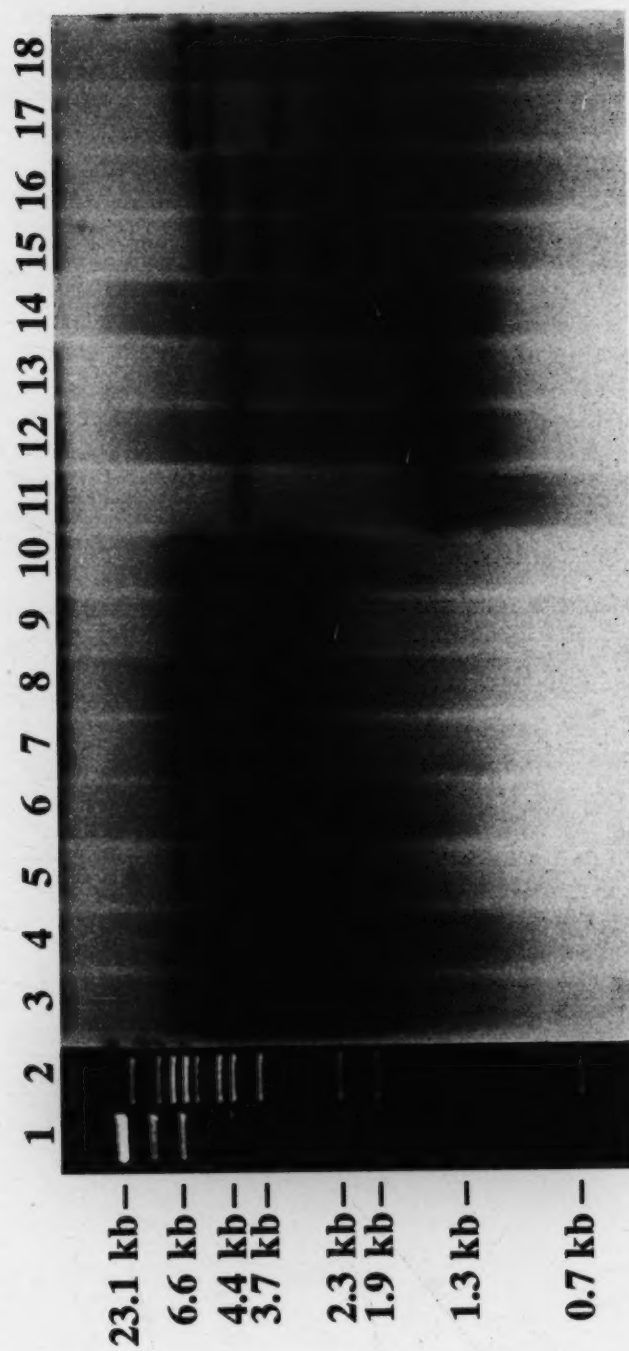


Figure 4.13. Southern Hybridization Analysis of *H-3* in *Brassica rapa* L. var. *oleifera* cv. Reward and *Brassica rapa* var. *pekinensis* (bok choy).

Genomic DNA from *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) was isolated from leaves. Two different plants from both *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were used to isolate two separate DNA samples for each variety. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *H-3* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested *B. rapa* L. var. *oleifera* cv. Reward genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested *B. rapa* var. *pekinensis* (bok choy) genomic DNA.

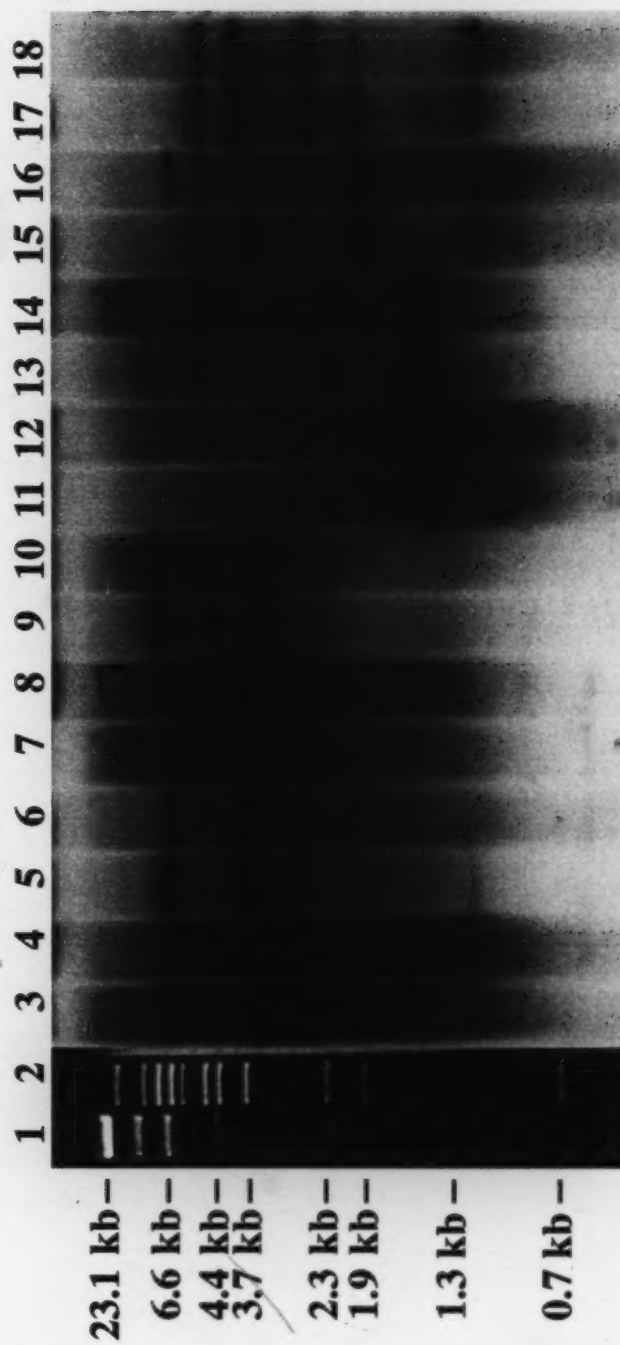


Figure 4.14. Southern Hybridization Analysis of *H-3* in *Brassica oleracea* var. *acephala* (kale) cv. Dwarf Green Curled Scotch and *Brassica oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese.

Genomic DNA from *B. oleracea* var. *acephala* (kale) cv. Dwarf Green Curled Scotch and *B. oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese was isolated from leaves. Two different plants from both kale and broccoli were used to isolate two separate DNA samples for each variety. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *H-3* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested kale genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested broccoli genomic DNA.

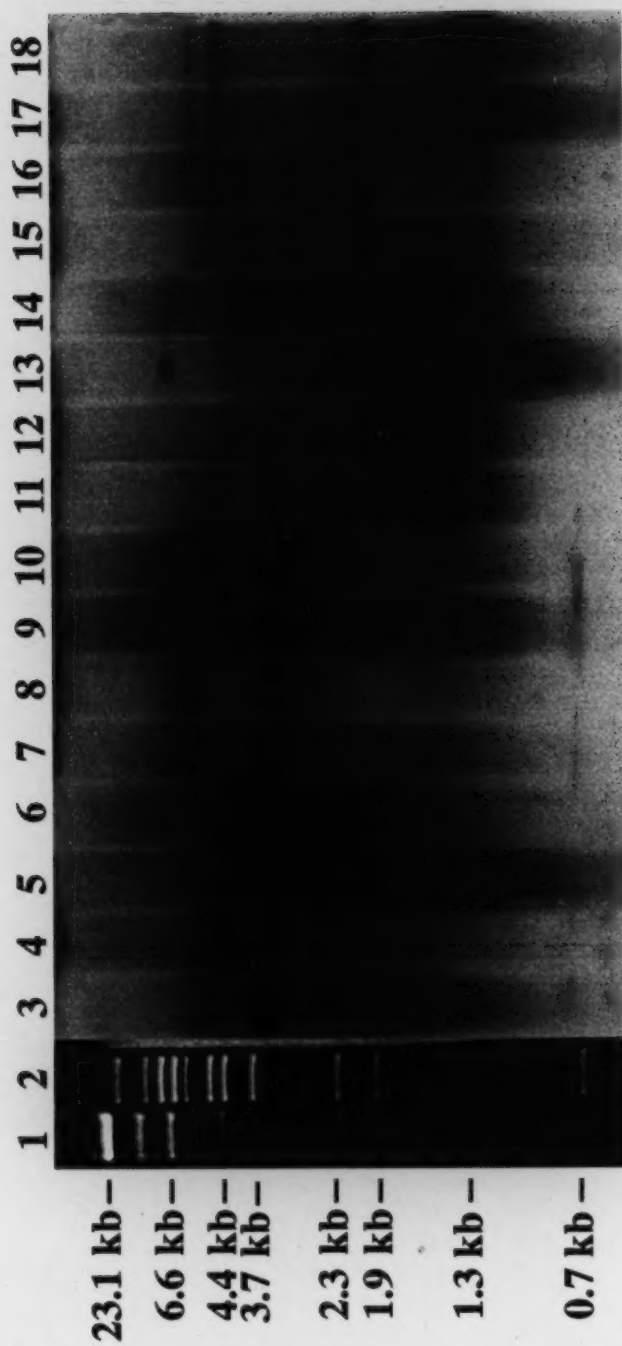


Figure 4.15. Southern Hybridization Analysis of *H-3* in *Brassica nigra* accession PI 271444 (Indian) and *Brassica nigra* accession PI 273642 (Ethiopian).

Genomic DNA from *B. nigra* accession PI 271444 (Indian) and *B. nigra* accession PI 273642 (Ethiopian) was isolated from leaves. Two different plants from both *B. nigra* accession PI 271444 (Indian) and *B. nigra* accession PI 273642 (Ethiopian) were used to isolate two separate DNA samples for each accession. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *H-3* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested *B. nigra* accession PI 273642 (Ethiopian) genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested *B. nigra* accession PI 271444 (Indian) genomic DNA.

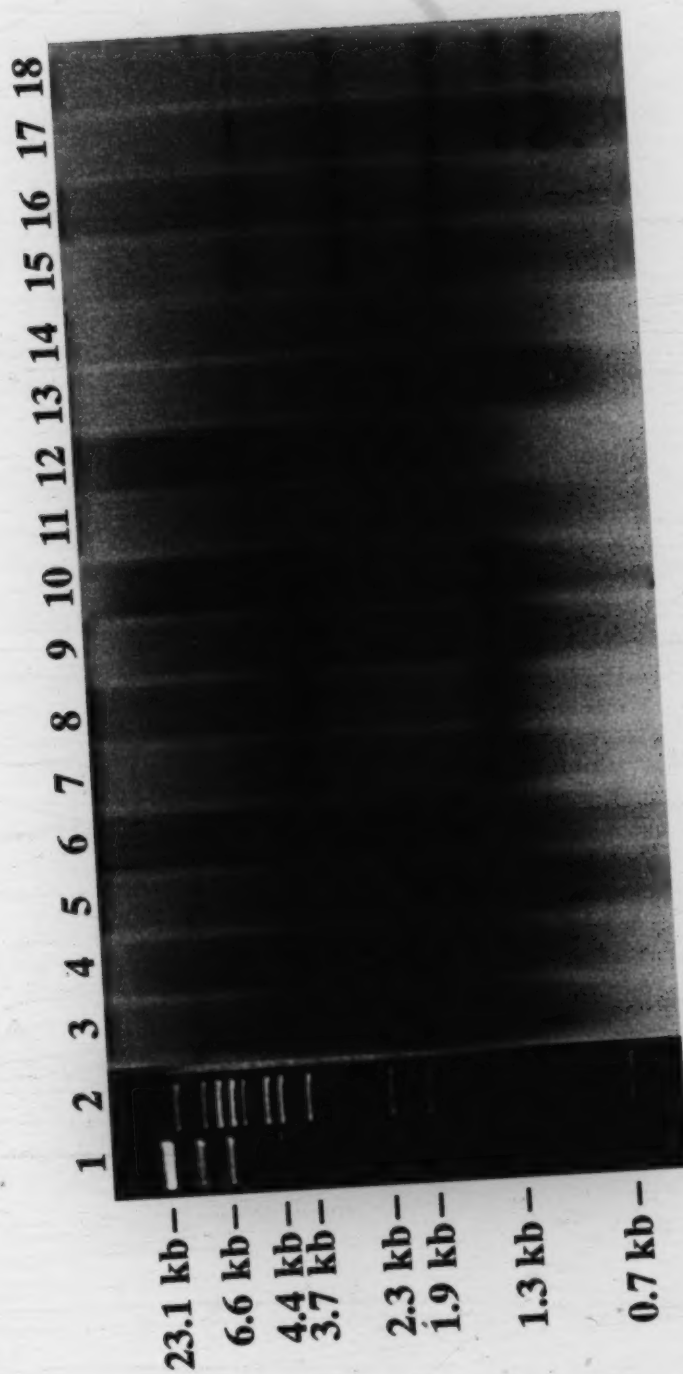


Figure 4.16. Southern Hybridization Analysis of *RPS2* in *Brassica napus* L. cv. Polo and *Brassica napus* L. cv. Westar.

Genomic DNA from *B. napus* L. cv. Polo and *B. napus* L. cv. Westar was isolated from leaves. Two different plants from both *B. napus* L. cv. Polo and *B. napus* L. cv. Westar were used to isolate two separate DNA samples for each cultivar. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen *Plus* (DuPont) membrane, and hybridized with a ³²P-labelled *RPS2* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested *B. napus* L. cv. Westar genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested *B. napus* L. cv. Polo genomic DNA.



Figure 4.17. Southern Hybridization Analysis of *RPS2* in *Brassica rapa* L. var. *oleifera* cv. Reward and *Brassica rapa* var. *pekinensis* (bok choy).

Genomic DNA from *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) was isolated from leaves. Two different plants from both *B. rapa* L. var. *oleifera* cv. Reward and *B. rapa* var. *pekinensis* (bok choy) were used to isolate two separate DNA samples for each variety. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *RPS2* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3 through 6 contain DNAs digested with *Bam*HI, lanes 7 through 10 contain DNAs digested with *Eco*RI, lanes 11 through 14 contain DNAs digested with *Eco*RV, and lanes 15 through 18 contain DNAs digested with *Hind*III. Lanes 3, 4, 7, 8, 11, 12, 15, and 16 contain digested *B. rapa* L. var. *oleifera* cv. Reward genomic DNA. Lanes 5, 6, 9, 10, 13, 14, 17, and 18 contain digested *B. rapa* var. *pekinensis* (bok choy) genomic DNA.



Figure 4.18. Southern Hybridization Analysis of *RPS2* in *Brassica oleracea* var. *acephala* (kale) cv. Dwarf Green Curled Scotch and *Brassica oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese.

Genomic DNA from *B. oleracea* var. *acephala* (kale) cv. Dwarf Green Curled Scotch and *B. oleracea* var. *italica* (broccoli) cv. Italian Green Sprouting Calabrese was isolated from leaves. Two different plants from both kale and broccoli were used to isolate two separate DNA samples for each variety. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *RPS2* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3, 7, 11, and 15 contain DNAs digested with *Bam*HI. Lanes 4, 8, 12, and 16 contain DNAs digested with *Eco*RI. Lanes 5, 9, 13, and 17 contain DNAs digested with *Eco*RV. Lanes 6, 10, 14, and 18 contain DNAs digested with *Hind*III. Lanes 3 through 10 contain digested kale genomic DNA. Lanes 11 through 18 contain digested broccoli genomic DNA.



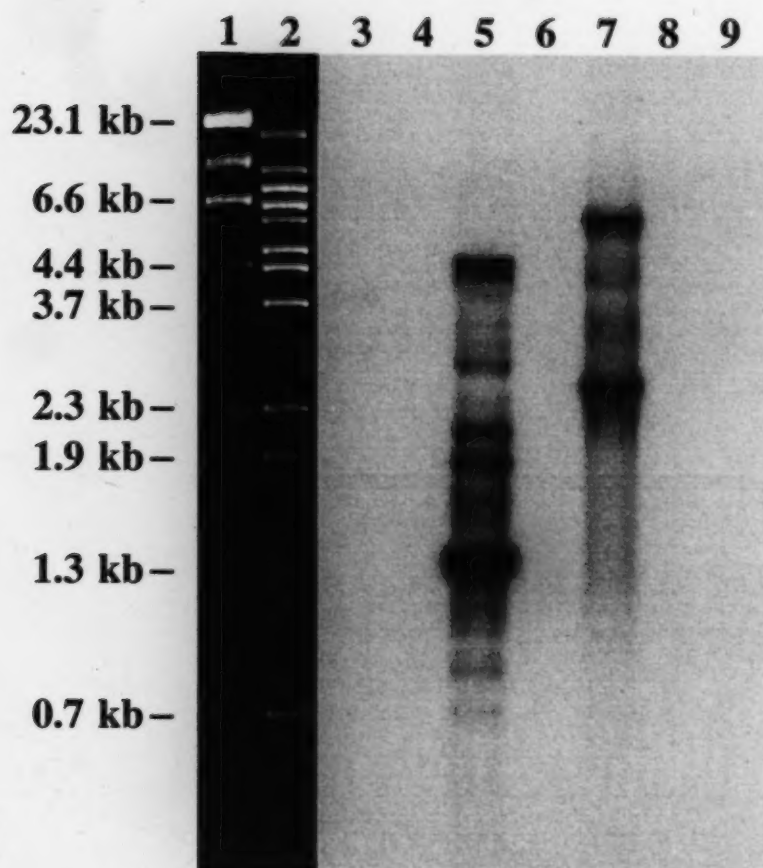
Figure 4.19. Southern Hybridization Analysis of *RPS2* in *Brassica nigra* accession PI 271444 (Indian) and *Brassica nigra* accession PI 273642 (Ethiopian).

Genomic DNA from *B. nigra* accession PI 271444 (Indian) and *B. nigra* accession PI 273642 (Ethiopian) was isolated from leaves. Two different plants from both *B. nigra* accession PI 271444 (Indian) and *B. nigra* accession PI 273642 (Ethiopian) were used to isolate two separate DNA samples for each accession. The DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III. Following digestion, 6 µg aliquots of the DNAs digested with the various restriction endonucleases were separated by electrophoresis through a 0.8% agarose gel, vacuum blotted onto a GeneScreen Plus (DuPont) membrane, and hybridized with a ³²P-labelled *RPS2* probe. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 3, 7, 11, and 15 contain DNAs digested with *Bam*HI. Lanes 4, 8, 12, and 16 contain DNAs digested with *Eco*RI. Lanes 5, 9, 13, and 17 contain DNAs digested with *Eco*RV. Lanes 6, 10, 14, and 18 contain DNAs digested with *Hind*III. Lanes 3 through 10 contain digested *B. nigra* accession PI 273642 (Ethiopian) genomic DNA. Lanes 11 through 18 contain digested *B. nigra* accession PI 271444 (Indian) genomic DNA.



Figure 4.20. *Fen* and *Pto* Southern Hybridization Control Blot.

Approximately 10 genomic equivalents of *H-3*, *Fen* (Martin et al., 1994), *Pto* (Martin et al., 1993a), and *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) alone; approximately 10 genomic equivalents of *H-3*, *Fen*, *Pto*, and *RPS2* in their linearized vectors; and 6 µg of sheared herring sperm DNA were separated by electrophoresis through a 0.8% agarose gel. The DNAs were then vacuum blotted onto a GeneScreen Plus (DuPont) membrane and hybridized with a ³²P-labelled *Fen* probe at 55°C as described in Materials and Methods. Lanes 1 and 2 contain a lambda *Hind*III digest (New England Biolabs) and a lambda *Bst*EII digest (New England Biolabs), respectively. These lambda digests were used as DNA size markers. Lanes 4, 6, and 8 are empty. Lane 3 contains *H-3*, *H-3* in its linearized pGEM-T (Promega) vector, and sheared herring sperm DNA. Lane 5 contains *Fen*, *Fen* in its linearized pCDNA II (Invitrogen) vector, and sheared herring sperm DNA. Lane 7 contains *Pto*, *Pto* in its linearized pCDNA II (Invitrogen) vector, and sheared herring sperm DNA. Lane 9 contains *RPS2*, *RPS2* in its linearized pBluescript KS+ (Stratagene) vector, and sheared herring sperm DNA. *H-3* is 540 bp, *Fen* is 1.3 kb, *Pto* is 2.4 kb, and *RPS2* is 2.73 kb. The pGEM-T (Promega) vector is 3 kb, the pCDNA II (Invitrogen) vector is 3 kb, and the pBluescript KS+ (Stratagene) vector is 2.96 kb.



5. DISCUSSION

5.1 PATHOGENICITY TESTS

It has never been established that a specific, known pathovar of *P. syringae* is the causal agent of disease in canola. That is, taxonomically identified *P. syringae* pathovars are not known to be phytopathogenic on canola. Consequently, one aspect of this present study was to provide evidence that known pathovars of *P. syringae* are infectious agents of *B. napus* and *B. rapa*.

The data presented in this thesis clearly show that *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000; Cuppels, 1986) is virulent on both *B. napus* L. and *B. rapa* L. var. *oleifera* (Tables 4.1 and 4.2). Moreover, the 9 bacterial isolates that were recovered from infected *B. napus* L. cv. Westar leaf tissue were conclusively proven to be *Pst* DC3000. These results were obtained by analyzing the bacterial isolates with numerous morphological, cultural, physiological, biochemical, hypersensitivity, pathogenicity, coronatine bioassay, and plasmid profile tests. All of these tests identified the 9 bacterial isolates as fluorescent pseudomonads and, in particular, as *Pst* DC3000. By experimentally satisfying all of Koch's postulates (Table 4.3; Agrios, 1988; Dickinson and Lucas, 1982; Nester et al., 1995), I have established that *Pst* DC3000 is a causal agent of disease in *B. napus* L. cv. Westar (Figure 4.2). This was a significant finding since tomato is the only common host of *P. syringae* pv. *tomato* (Agrios, 1988; Palleroni, 1984; Bradbury, 1986).

However, it was not completely unexpected that *Pst* DC3000 would be infectious on *B. napus* L. and *B. rapa* L. var. *oleifera* because *P. syringae* pv. *tomato* is closely related to the crucifer pathogen, *P. syringae* pv. *maculicola* (Hildebrand et al., 1982; Denny, 1988b; Davis et al., 1991; Henderson et al., 1992; Takikawa et al., 1994; Cuppels and Ainsworth, 1995). The host plants of *P. syringae* pv. *maculicola* are all in the family *Cruciferae* and most are in the genus *Brassica* (Bradbury, 1986).

The data presented in this thesis lend support to the proposal that at least *Pst* DC3000 should be taxonomically grouped with *P. syringae* pv. *maculicola*. That is,

the data presented here definitively showed that *Pst* DC3000, like *P. syringae* pv. *maculicola* (Wiebe and Campbell, 1993), is an infectious agent of the crucifer, *B. napus* L. cv. Westar. This taxonomic move for *Pst* DC3000 is also supported by the fact that *Pst* DC3000 has been shown to infect various other members of the *Cruciferae* family (Davis et al., 1991; Henderson et al., 1992) even though it is classified as a *P. syringae* pv. *tomato* strain. Moreover, Cuppels and Ainsworth (1995) have shown with pathogenicity and carbon utilization tests that *Pst* DC3000 more closely resembles strains of *P. syringae* pv. *maculicola* than other *P. syringae* pv. *tomato* strains.

P. syringae pv. *maculicola* strain 84-59 (Wiebe and Campbell, 1993) was shown to have almost the same pathogenic capacity on *B. napus* L. and *B. rapa* L. var. *oleifera* as *Pst* DC3000 (Tables 4.1 and 4.2). Based on this infectivity of *P. syringae* pv. *maculicola* strain 84-59 to the various *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars examined (Tables 4.1 and 4.2), it can be said that canola is probably a host for *P. syringae* pv. *maculicola*. This conclusion is further supported by the fact that Wiebe and Campbell (1993) were able to show that three different *P. syringae* pv. *maculicola* strains (4981, 84-67, and 83-05) were pathogenic on *B. napus* L. cv. Westar and two different *P. syringae* pv. *maculicola* strains (4981 and 84-67) were pathogenic on *B. rapa* L. var. *oleifera* cv. Candle. Consequently, it is likely that *P. syringae* pv. *maculicola* may be a causal agent of taxonomically uncharacterized and agronomically unimportant bacterial diseases of canola. Even if *P. syringae* pv. *maculicola* is not responsible for natural canola diseases, canola crops and debris could definitely act as a reservoirs for this phytopathogen.

However, the data obtained from the *P. syringae* pv. *maculicola* strain 84-59 pathogenicity tests must be taken with caution since an inoculum concentration of 1×10^8 cfu/mL was used in these tests. An inoculum level of 1×10^8 cfu/mL is a high dosage to use for pathogenicity tests and may cause an assortment of reactions such as necrosis, discoloration, eruptions, and HR on non-host plants which can lead to a misinterpretation of host range (Hildebrand et al., 1988). Therefore, it is possible,

although unlikely, that *B. napus* L. and *B. rapa* L. var. *oleifera* are not true hosts of *P. syringae* pv. *maculicola* strain 84-59. A misinterpretation of host range is unlikely since inoculum levels of 1×10^7 cfu/mL are routinely used for *P. syringae* pv. *maculicola* pathogenicity tests (Wiebe and Campbell, 1993; Cuppels and Ainsworth, 1995) and inoculum levels of 1×10^8 cfu/mL are commonly used for various *Pseudomonas* pathogenicity tests (Jones et al., 1981; Martin et al., 1991; Whalen et al., 1991; Carland and Staskawicz, 1993; Kunkel et al., 1993; Bent et al., 1994; Salmeron et al., 1994; Rommens et al., 1995a; Gopalan et al., 1996).

5.2 CANOLA LACKS R GENES CAPABLE OF RECOGNIZING *avrPto* AND *avrRpt2*

It was determined in this study that *P. syringae* pv. *tomato* strains T1 (Denny, 1988a) and T1(pPtE6) (Ronald et al., 1992) were not pathogenic on the majority of *B. napus* L. cultivars examined. However, they were weakly virulent on all of the *B. rapa* L. var. *oleifera* cultivars analyzed in this study. What is significant about these findings was that none of the *B. napus* L. or *B. rapa* L. var. *oleifera* cultivars were resistant to *P. syringae* pv. *tomato* strain T1(pPtE6) and susceptible to *P. syringae* pv. *tomato* strains T1. They were either resistant or slightly susceptible to both strains. There was no detectable difference in symptom formation when the canola cultivars were inoculated with either *P. syringae* pv. *syringae* strain T1 or *P. syringae* pv. *syringae* strain T1(pPtE6).

P. syringae pv. *tomato* strain T1(pPtE6) is actually the natural *P. syringae* pv. *tomato* strain T1 expressing an *avrPto* bacterial avirulence transgene (Ronald et al., 1992). Consequently, the *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars analyzed in this study (Tables 3.3 and 3.4) do not possess a disease resistance gene that is capable of recognizing the *avrPto* gene product. Since *Pto* confers resistance only to *P. syringae* pv. *syringae* races that express *avrPto* (Ronald et al., 1992), *H-3* must represent a *Pto* homolog that recognizes a different avirulence gene product than *Pto*.

Similarly, none of the *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars

analyzed in this study (Tables 3.3 and 3.4) were resistant to *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω) (Whalen et al., 1991) and susceptible to *P. syringae* pv. *tomato* strain DC3000(pLH12) (Whalen et al., 1991). All of these cultivars were susceptible to varying degrees to both strains (Tables 4.1 and 4.2). None of the cultivars examined possessed a resistant phenotype to *P. syringae* pv. *tomato* strain DC3000(pLH12).

P. syringae pv. *tomato* strain DC3000(pLH12) is, in fact, *Pst* DC3000 expressing the avirulence gene, *avrRpt2* (Whalen et al., 1991). *P. syringae* pv. *tomato* strain DC3000(pLH12 Ω) is essentially identical to the natural *Pst* DC3000 phytopathogen because it contains a disrupted, nonfunctional *avrRpt2* gene (Whalen et al., 1991). Although RFLP analysis demonstrated that *B. napus* L. and *B. rapa* L. var. *oleifera* possess an *RPS2* homolog (section 4.8), these pathogenicity studies indicate that this homolog is incapable of recognizing *avrRpt2*. Importantly, the *RPS2* resistance gene was cloned in *A. thaliana* and it confers resistance against isolates of the bacterial pathogen, *P. syringae*, that express the avirulence gene, *avrRpt2* (Bent et al., 1994; Mindrinos et al., 1994). Therefore, the *Brassica* *RPS2* homolog must recognize a different avirulence gene product than the one produced by *avrRpt2*.

5.3 FENTHION AND MALATHION SCREENING EXPERIMENTS

The reaction of the crop brassicas to both fenthion and malathion proved to be generally uninformative. All of the crop brassicas exposed to fenthion developed necrotic lesions (Tables 4.4 and 4.5). Although the crop brassicas were sensitive to varying degrees, none of them exhibited a distinct phenotypic response. That is, all of the accessions and cultivars of the various *Brassica* species and varieties analyzed displayed substantial phenotypic ranges. Consequently, within any of the *Brassica* species it would have been impossible to demonstrate cosegregation of *H-3* with a fenthion phenotypic response. Nevertheless, these data indicate that all of the crop brassicas examined have receptors that are capable of recognizing the fenthion molecule and, therefore, likely possess *Fen* gene (Martin et al., 1994) homologs.

The *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars screened with malathion were essentially insensitive to this insecticide. This insensitivity indicates that malathion may have to be applied at extremely high concentrations such as those used in controlling insect pests to produce necrotic lesions on canola. It is also reasonable to assume that canola cultivars do not possess malathion receptors. The malathion molecule may be too structurally different from fenthion for a fenthion receptor to recognize it. This hypothesis is supported by the fact that malathion did not produce any necrotic lesions on fenthion sensitive, *L. esculentum* cv. Rio Grande-PtoR (*Fen/Fen*) plants.

5.4 PCR AMPLIFICATION OF H-3

Interestingly, the PCR products of the three *B. napus* breeding lines that were generated with primer combination I and primer BKE-3 alone were identical in appearance (Figure 4.6 - lanes 3 and 7). This finding implied that the PCR products generated with primer combination I were, in fact, products amplified only by the BKE-3 primer. This scenario could have arisen if there were no BKE-1 and BKE-3 binding sites sufficiently close to each other to allow DNA amplification to occur. There may also have been no binding sites for the BKE-1 primer in the *B. napus* L. genome. The PCR products that were generated with primer combination I could, therefore, have been amplified by BKE-3 alone as would be seen in a random amplified polymorphic DNA (RAPD) reaction (Williams et al., 1990).

It is also possible that the PCR products were not generated by a single primer since the BKE-3 primer is actually a 48-fold degenerate PCR primer. The low stringency conditions of the PCR may have permitted the binding of some of the 48 BKE-3 primers to multiple sites within the genomes of the *B. napus* L. breeding lines. In either case, the PCR products generated by primer combination I were not specific to BKE-1 and BKE-3 amplification and, therefore, could not reliably be considered to constitute *Pto* homologs. As a result, only the PCR amplification product of the expected size (540 bp fragment) generated by primer combination II was analyzed further.

5.5 ANALYSES OF H-3: A *Brassica napus* L. Pto HOMOLOG?

Since *H-3* contained an uninterrupted ORF and its deduced amino acid sequence was shown to have the highest similarity with Pto (Martin et al., 1993a), it is likely that *H-3* encodes a disease resistance gene product. Nevertheless, I was unable to verify that *H-3* encodes a Pto *R* gene homolog since I could not demonstrate the cosegregation of *H-3* with a phenotypically defined disease resistance response.

The phenotypic responses and *Pst* DC3000 growth curves of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in replicate one of the determinative pathogenicity study (section 3.5.3) supported the concept that *B. napus* L. cv. Polo was a resistant cultivar (Figure 4.1 A and B; Figure 4.3 A) and *B. napus* L. cv. Westar was a susceptible cultivar (Figures 4.2 and 4.3 A). Moreover, RFLP analysis of *B. napus* L. cv. Polo and *B. napus* L. cv. Westar was performed after this pathogenicity replicate and this analysis showed that numerous polymorphisms existed between these two cultivars when they were probed with *H-3* (Figure 4.12). These polymorphisms proved that these two cultivars were genetically different at the *H-3* locus. Therefore, I initiated the breeding program between *B. napus* L. cv. Polo and *B. napus* L. cv. Westar in order to obtain F_1 , F_2 , and backcross generations for use in segregation analysis with *H-3*. Unfortunately, the next two replicates of the determinative pathogenicity study revealed that *B. napus* L. cv. Polo exhibited extensive variability towards *Pst* DC3000 infection (Figures 4.1 and 4.3).

Although I was able to demonstrate that *H-3* comprises an uninterrupted ORF that contains Pto sequence motifs internal to those used as priming sites, I was unable to provide all of the genetic criteria necessary to establish that *H-3* is a functional disease resistance gene. Due to the variability seen in *B. napus* L. cv. Polo, I could not use the reciprocal Polo x Westar F_1 , F_2 , and backcross generations to establish that *H-3* segregates with phenotypically defined *Pst* DC3000 resistance. Consequently, I did not attempt transgenic complementation or mutant analysis to prove that the PCR-derived *H-3* sequence was a resistance gene. Since transgenic complementation or mutant analysis studies must be completed to prove that a sequence derived from PCR

is a resistance gene (Michelmore, 1996), I cannot be assured that *H-3* is a functional resistance gene.

Regardless, it is plausible that *H-3* does represent a *B. napus* *Pto* homolog since the *H-3* polypeptide and *Pto* (Martin et al., 1993a) contain the same serine/threonine kinase domains and invariant residues (Figures 4.9 and 4.10). The *H-3* polypeptide also shares 68% identity and 82% similarity with *Pto* and it possess a region that is at least 71% identical and 88% similar to the region in *Pto* that is required for the interaction with *AvrPto* (Figure 4.11; Scofield et al., 1996; Tang et al., 1996). This similarity to the *AvrPto*-interaction site in *Pto* is very significant since this *Pto* region is absolutely essential for the elicitor-receptor mechanism of gene-for-gene incompatibility in tomato (Scofield et al., 1996; Tang et al., 1996). It is likely that conserved resistance mechanisms exist among canola and tomato since the *H-3* and *Pto* polypeptides share such a high degree of similarity.

However, *H-3* does not appear to confer the same type of resistance to canola that *Pto* confers to tomato. The tomato *Pto* gene confers resistance against strains of *P. syringae* pv. *tomato* that possess the avirulence gene, *avrPto* (Ronald et al., 1992). As already stated, the *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars analyzed in this study were incapable of recognizing *avrPto* (section 5.2). Even though *P. syringae* pv. *tomato* was shown to infect canola, the *Brassica H-3* gene does not confer resistance to infection by strains expressing *avrPto*. If *H-3* is a functional *R* gene it must recognize another avirulence gene product.

It is possible that *H-3* represents a loss-of-function allele that resulted from point mutations that altered the amino acid sequence or created nonsense mutations. Since the entire *H-3* gene was not cloned, I cannot be assured that nonsense mutations do not exist within *H-3* and, thus, *H-3* could represent a pseudogene. It is also feasible that *H-3* may be a functional kinase but, like *pto* (Jia et al., 1997), it may not be able to confer disease resistance. *H-3* may have differences in its amino acid sequence that generate hindrances in signal molecule recognition or protein-protein interactions.

5.6 A NEW CLASS OF SERINE/THREONINE PROTEIN KINASES?

H-3 likely encodes a serine/threonine protein kinase (Figures 4.9 and 4.10; Table 4.7). The *H-3* polypeptide contains many characteristics that are highly specific to serine/threonine kinases (Figure 4.10) and the majority of the most similar proteins to *H-3* were identified as plant serine/threonine protein kinases.

The *H-3* polypeptide shared the most similarity with *Pto* (Martin et al., 1993a), *pto* (Jia et al., 1997), *CrRLK1* (Schulze-Muth et al., 1996), 2642445 (GenBank accession number AC002391), *fen* (Jia et al., 1997), and *Fen* (Martin et al., 1994). These six proteins were significantly more similar to *H-3* than all of the other proteins identified by the BLASTX search (Altschul et al., 1990; Gish and States, 1993). Specifically, the *Fen* protein was the sixth most similar protein to *H-3* and it had an *E* value of 1.0×10^{-60} (Table 4.7) whereas the seventh most similar protein to *H-3* had a substantially lower *E* value of 2.9×10^{-39} . As a result, *H-3* and the six most similar proteins are not only very similar to each other but they are also distinct from other proteins.

Eukaryotic protein kinases make up a large superfamily and they are classified into various groups, subgroups, families, and subfamilies (Hanks and Hunter, 1995). Plant kinases are currently grouped into ten categories (Hanks and Hunter, 1995). Hanks and Hunter (1995) have included *Pto* (Martin et al., 1993a) in the flowering plant putative receptor kinase family.

However, my current analysis of the plant serine/threonine kinases indicates that *Pto* belongs in the same family as *H-3*, *pto*, *CrRLK1*, 2642445, *fen*, and *Fen*. *Pto* shares much more identity with these polypeptides than it does with the proteins included in the flowering plant putative receptor kinase family (Hanks and Hunter, 1995). Consequently, it is reasonable to assume that *H-3*, *Pto*, *pto*, *CrRLK1*, 2642445, *fen*, and *Fen* constitute a distinct, dicotyledonous plant serine/threonine protein kinase family. The classification of these proteins as a new kinase family is also supported by the similar functional roles of these proteins.

Pto is a tomato disease resistance gene product that recognizes an avirulence gene product (Martin et al., 1993a; Scofield et al., 1996; Tang et al., 1996) and *pto* is

a functional protein kinase encoded by an allele of *Pto* (Jia et al., 1997). The periwinkle CrRLK1 protein is functional receptor-like kinase that likely has a role in the perception and transduction of external signals (Schulze-Muth et al., 1996). Fen is a tomato protein kinase that is highly homologous to *Pto* and it recognizes the insecticide, fenthion (Martin et al., 1994). The fen protein is analogous to *pto* in the sense that it is a functional kinase encoded by an allele of *Fen* (Jia et al., 1997). Finally, 2642445 (GenBank accession number AC002391) is the deduced polypeptide of an *A. thaliana* genomic clone which appears to be a putative serine/threonine protein kinase. Hence, all of these proteins are involved in signal detection and transduction in dicotyledonous plants.

5.7 RFLP ANALYSIS OF THE CROP BRASSICAS

One of the most significant findings of the RFLP analysis was that multiple bands were detected in all of the crop brassicas when they were hybridized with *H-3* (Figures 4.12 - 4.15). The presence of multiple RFLP bands implies that an *H-3* gene family may be present in the crop brassicas (Martin et al., 1993a). The possibility of an *H-3* gene family in the crop brassicas provides support to the hypothesis that *H-3* represents a *Pto* homolog in the *Brassica* species because *Pto* also belongs to a gene family containing at least six members (Martin et al., 1993a; Martin et al., 1994; Jia et al., 1997). Thus, it is feasible that there are other *Brassica* genes with structural and, perhaps, functional similarity to other members of the *Pto* gene family. The other principal finding of the RFLP analysis was that an *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) homolog was shown to be present in *B. napus* L., *B. rapa*, *B. oleracea*, and *B. nigra* (Figures 4.16 - 4.19).

The RFLP analysis using *H-3* and *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) demonstrated the genomic relationships of the crop brassicas. The *B. napus* L. RFLP banding patterns appeared to be composed of a mixture of bands from both *B. oleracea* and *B. rapa* (Figures 4.12 - 4.14 and 4.16 - 4.18) and there were no common RFLP bands found between *B. napus* L. and *B. nigra* (Figures 4.12, 4.15, 4.16, and

4.19). These data comply with the known evolutionary relationship of the crop brassicas. That is, *B. napus* L. ($2n = 38$; AACC) is an amphidiploid that arose via interspecific hybridization between the elementary diploids, *B. rapa* ($2n = 20$; AA) and *B. oleracea* ($2n = 18$; CC). In addition, *B. napus* L. does not possess the B genome of *B. nigra* ($2n = 16$; BB) (Labana and Gutpa, 1993; Figure 2.3).

There were *H-3* and *RPS2* RFLP bands that were distinct to the *B. napus* L. cultivars examined. This finding indicates that the *H-3* and *RPS2* genes in *B. napus* L. have undergone evolutionary changes from the *H-3* and *RPS2* genes present in *B. oleracea* and *B. rapa*. Deletions, insertions, or point mutations are the likely causes of the changes in the *B. napus* L. genes.

An interesting observation of the *H-3* and *RPS2* RFLP analysis was the finding that DNA digestion with some restriction enzymes resulted in nonspecific background noise. This lack of signal detection with some restriction enzymes indicated that the DNA was cut into very large fragments with these particular enzymes. Due to inefficient transfer of very large DNA fragments (≥ 50 kbp) during Southern blotting (Sambrook et al., 1989; Selden, 1989), these fragments would not have been transferred to the hybridization membranes. Hence, it would be impossible to detect any RFLP bands with the *H-3* and *RPS2* probes when the *Brassica* species were digested with these restriction enzymes.

The RFLP analysis also established that both the *Pto* (Martin et al., 1993a) and *Fen* (Martin et al., 1994) genes were too divergent from *H-3* to detect this homolog in the *Brassica* species. The lack of any positive signals detected by the *Pto* and *Fen* probes was not striking as *H-3* only had 63% nucleic acid identity with *Pto* and 58% nucleic acid identity with *Fen*. This sequence divergence would prevent *Pto* and *Fen* signal detection in the crop brassicas. Consequently, *H-3* and *Pto*; and *H-3* and *Fen* are only highly similar at the amino acid level. This polypeptide conservation suggests that disease resistance may be evolutionarily conserved among taxonomically divergent plants at the functional level of proteins.

6. SUMMARY AND CONCLUSION

The data presented in this thesis clearly establish that the tomato pathogen, *Pst* DC3000, causes an infectious disease in the crucifer, *B. napus* L. cv. Westar. It was also revealed that *Pst* DC3000 and the crucifer pathogen, *P. syringae* pv. *maculicola* strain 84-59, exhibited essentially the same virulence on all of the *B. napus* L. and *B. rapa* L. var. *oleifera* cultivars examined in this study. These two findings lend support to the concept that *P. syringae* pathovars *maculicola* and *tomato* may constitute a single genetic group (Palleroni, 1984; Henderson et al., 1992). However, this *Pseudomonas* data only represents a pathogenicity analysis and the genetic makeup of strains should not be determined solely on the basis of pathogenicity (Henderson et al., 1992). Therefore, until genetic and biochemical analyses have taxonomically clarified the *P. syringae* pathovars *maculicola* and *tomato*, *Pst* DC3000 should remain within *P. syringae* pv. *tomato* and the two pathovars should remain distinct (Cuppels and Ainsworth, 1995).

In this study I used degenerate oligonucleotide primers based on the conserved catalytic subdomains of serine/threonine protein kinases and *Pto* (Martin et al., 1993a) to amplify a catalytic subdomain-containing *Pto* homolog from *B. napus* L. This *B. napus* L. PCR product contained an uninterrupted ORF that had strong sequence identity with known plant serine/threonine kinases. This PCR product was called *H-3* and it showed the strongest nucleic acid identity with the *Pto* disease resistance gene (Martin et al., 1993a). The deduced amino acid sequence also shared the highest percent identity and similarity with the *Pto* polypeptide sequence. RFLP analysis of the crop brassicas indicated that they may possess an *H-3* gene family. This finding was significant since *Pto* also belongs to gene family containing at least six members (Martin et al., 1993a; Martin et al., 1994; Jia et al., 1997). Consequently, *H-3* likely represents a *Pto* homolog within the *Brassica* genus.

Sequence analysis revealed that the *H-3* polypeptide and six other serine/threonine plant protein kinases are not only exceptionally similar to each other but they are also distinct from other proteins. This group of plant protein kinases

includes Pto (Martin et al., 1993a), pto (Jia et al., 1997), CrRLK1 (Schulze-Muth et al., 1996), 2642445 (GenBank accession number AC002391), fen (Jia et al., 1997), and Fen (Martin et al., 1994). The similarity and uniqueness of these proteins indicates that they may constitute a new dicotyledonous plant serine/threonine protein kinase family.

These findings demonstrate that conserved sequences from resistance genes can be employed in a PCR-based approach to identify candidate disease resistance genes in diverse plant taxa. This PCR-based approach has also been utilized by three other groups to amplify sequences homologous to the nucleotide binding sites of the *L6*, *N*, and *RPS2* resistance genes (Kanazin et al., 1996; Leister et al., 1996b; Yu et al., 1996). Thus, the application of heterologous PCR to identify and clone plant resistance genes has tremendous potential. Resistance gene homologs will have immediate benefits since they represent ideal molecular markers for disease resistance (Michelmore, 1996).

My results and those of Kanazin et al. (1996), Leister et al. (1996b), and Yu et al. (1996) show that plant resistance genes are evolutionarily conserved throughout the plant kingdom. These results also reveal that conserved resistance mechanisms exist among plants. These concepts are further supported by the fact that I was able to identify an *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) homolog in the crop brassicas with a stringent RFLP analysis.

Nevertheless, genetic criteria such as segregation analysis, mutant analysis, or transgenic complementation must be used to prove that *H-3* and the *RPS2* homolog encode functional *Brassica* disease resistance genes. Until these analyses have been completed these resistance gene homologs cannot be considered functional disease resistance genes (Michelmore, 1996). *H-3* and the *RPS2* homolog may be pseudogenes within the crop brassicas.

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8. APPENDICES

8.1 VACUUM BLOTTING PROTOCOL

This procedure is a modified version of the protocol given in the Bio-Rad model 785 vacuum blotter instruction manual (Bio-Rad Laboratories, Richmond, CA, USA).

- (1) Vacuum blotting is done at 5 in. Hg.
- (2) Depurinate the DNA by pouring 0.25 M HCl on top of the agarose gel so that it is completely covered. Depurinate for 15-20 min until the bromophenol dye front in the agarose gel turns yellow.
- (3) Remove any remaining 0.25 M HCl from the agarose gel and rinse briefly with denaturing solution (0.5 M NaOH, 1.5 M NaCl). Remove the denaturing solution.
- (4) Denature the DNA by pouring denaturing solution (0.5 M NaOH, 1.5 M NaCl) on top of the agarose gel so that it is completely covered. Denature for 20 min until the bromophenol blue dye front turns back to a blue colour.
- (5) Remove any remaining denaturing solution.
- (6) Neutralize the DNA by pouring neutralizing solution (0.5 M Tris, 3 M NaCl - pH 7.5) on top of the agarose gel so that it is completely covered. Neutralize for 15 min.
- (7) Remove any remaining neutralizing solution.
- (8) Transfer the DNA to the hybridization membrane by pouring 10x SSC (1.5 M NaCl, 0.15 M sodium citrate dihydrate) on top of the agarose gel so that it is completely covered. Transfer for 1 h making sure that the agarose gel is always completely covered with 10x SSC.
- (9) Remove any remaining 10x SSC, turn off the vacuum, and peel the agarose gel off the blotter. Carefully separate the hybridization membrane from the Whatman 3MM paper.
- (10) Agitate the hybridization membrane in 0.4 M NaOH for 1 min.
- (11) Agitate the hybridization membrane in 0.2 M Tris (pH 7.5), 2x SSC for 5 min.
- (12) Agitate the hybridization membrane in 2x SSC for 5 min.
- (13) Place the hybridization membrane between two pieces of clean Whatman 3MM paper and bake at 80°C for 2 h.
- (14) Store the baked hybridization membrane at room temperature until needed.